CLINICAL RELEVANCE OF TUMOUR INFILTRATING LYMPHOCYTES IN COLORECTAL CANCER

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1 SUMMARY

Background: Approximately 10-30% of colorectal cancers are characterized with distinctly high densities of tumour-infiltrating lymphocytes (TILS), including CD3+, cytotoxic CD8+, activated cytotoxic CD8+/Granzyme B (GZMB)+, and regulatory T (Treg) FOXP3+ cells. High densities of TILS in CRC have been associated with good prognosis. However, it has been unclear whether the longer survival associated with TILS is due solely to the anti-tumour immune response, or whether this phenotype also responds better to adjuvant treatment. Furthermore, the propensity for certain CRC patients to have high TILS is not well-understood.

Aims: This study aimed to: (i) evaluate the prognostic and predictive significance of TILS and its subtypes. (ii) assess the correlation between the densities of T cell subtypes in the peripheral blood and primary tumours in CRC patients; (iii) characterize the correlations between single nucleotide polymorphisms (SNPs) in *CD8a*, *GZMB* and *FOXP3* genes in CRC patients and respective gene expression and T cell densities in peripheral blood and tumours.

Methods: Tissue microarrays containing tumour samples from 439 CRC cases were immunohistochemically evaluated for the densities of CD3, CD8, Granzyme B (GZMB) and FOXP3-positive tumour-infiltrating lymphocytes. The prognostic significance of high CD3+, CD8+, CD8+GZMB+ and FOXP3+ cell density was evaluated in patients treated with surgery alone, while their predictive significance was estimated by comparing the survival of stage III patients treated with or without 5-Fluorouracil (5-FU)-based chemotherapy. Matched peripheral blood samples and tissue sections from 50 CRC patients were analyzed for their T cell densities by flow

cytometry and immunohistochemistry respectively. SNPs in *CD8a* (n=12), *GZMB* (n=22) and *FOXP3* (n=41) were genotyped by Sequenom MassARRAY iPLEX analysis, and examined for their correlation with respectively blood and tumour T cell densities and gene expression levels.

Results: High densities of CD3+, CD8+, CD8+GZMB+ and FOXP3+ cells were associated with better overall survival (hazard ratios (HRs) of 0.57-0.67) in univariate (each $P<0.05$) analysis. Stage III patients with high CD3+, CD8+ and CD8+GZMB+ cell densities and treated with chemotherapy had a better survival than patients treated by surgery alone (HR= 0.18, 0.26 and 0.26, respectively; each $P<0.01$). Patients with low densities also had a better survival from treatment, but to a lesser degree (HR= 0.55, 0.51 and 0.51, respectively; $P=0.11$, 0.05 and 0.06, respectively). The degree of inferiority was significant for CD3+ ($P=0.032$). Densities of each of the T cell subtypes in peripheral blood correlated with respective intra-tumour densities (all $P<0.05$). Eleven SNPs in blood and 3 in tumour samples were associated with respective T cell densities ($P<0.05$). The *GZMB* rs8192922 SNP was the only one associated with both blood and tumour densities, with higher densities of CD8+GZMB+ cells associated with the GG genotype.

Conclusions: The improved prognosis of CRC patients with TILS may in part be due to a beneficial interaction between TILS and chemotherapy. CRC patients with TILS may have heightened circulating T cell densities compared to those without TILS. The *GZMB* rs8192922 GG genotype may be a related factor in these heighted densities. Taken together, the results suggest less invasive genetic and/or peripheral blood monitoring of TILS, and consequent prognostication and prediction of 5-FU response in CRC patients, could be feasible. Future work to validate these findings is

required.

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4 INTRODUCTION

4.1 Colorectal Cancer

With almost one million new cases per year, colorectal cancer (CRC) accounts for 9.4% of all new cancer cases worldwide (Parkin 2004). The rates of progress of industrialization and urbanization have paralleled the rates of CRC (Labianca *et al.* 2010). During the period of 2003-2007, colorectal cancer was the first and second commonest cancers among Singaporean men and women, respectively. It was also one of the cancers with the highest mortality rate in both genders (SingaporeCancerRegistry 2003-2007). Older age, high intake of red meat, obesity and living with sedentary life style are factors that contribute to a high incidence of CRC in different degrees (Seow *et al.* 2002; Wong and Eu 2007).

Surgery remains the primary treatment for stage I to III colon cancer and adjuvant chemotherapy is used to reduce the risk of relapse and death in stage II-IV patients. Currently, chemotherapy is recommended post-operatively in high-risk stage II (Midgley and Kerr 2005) and stage III patients. However, it is debatable whether chemotherapy is needed in stage II patients since they have a 80% chance of being relapse-free (Gray *et al.* 2007).

Various clinicopathological and molecular parameters have been examined for their prognostic significance in CRC. Currently, TNM staging remains as the gold standard for prognosis. Other histopathological features such as vascular invasion, resection margin and tumour grade have also been well-studied for their associations with prognosis (Compton *et al.* 2000; Morris *et al.* 2006). Clinical parameters including obstruction and perforation at presentation (Steinberg *et al.* 1986), and pre-operative

carcinoembryonic antigen (CEA) levels (Wanebo *et al.* 1978) have been shown to have an independent influence on survival outcomes. In addition to histopathological and clinical factors, genetic alterations have been described to refine prognostic information and predict survival benefit derived from systemic treatment. The mutation status of *KRAS* (Andreyev *et al.* 1998; Andreyev *et al.* 2001) and *APC* (Lovig *et al.* 2002) and *TP53* (Russo *et al.* 2005), loss of heterozygocity (LOH) of chromosome 18q (Alhopuro *et al.* 2005; Popat and Houlston 2005) and the CpG island methylator phenotype (CIMP) (Ogino *et al.* 2009) have been shown to be associatied with CRC patient survival outcomes. However, none of these markers is in routine clinical use and more effort is needed to validate their reliability and consistency.

4.2 5-Fluorouracil-Based Chemotherapy

The antimetabolite, 5-Fluorouracil (5-FU) is a pyrimidine analog that was designed and synthesized by Charles Heidelberger in 1957 (Heidelberger *et al.* 1957). It has been widely used for treating a range of cancers, including colorectal and breast cancers, and cancers of the aerodigestive tract (Longley *et al.* 2003). It principally acts as a thymidylate synthase inhibitor and can be converted intracellularly to several active metabolites such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) (Longley *et al.* 2003). These metabolites disrupt RNA and DNA synthesis as well as the action of thymidine synthase. This leads to cell cycle arrest and apoptosis through disruption of DNA synthesis.

5-FU has been used for more than 40 years in the treatment of CRC, however, the overall response rate for 5-FU as a single agent in advanced CRC is only 10-15%

(Johnston and Kaye 2001). Therefore, important modulation strategies have been developed to increase the anti-cancer activity of 5-FU and prevent clinical resistance. The combination of 5-FU with other drugs including leucovorin, methotrexate (MTX), oxaliplatin and irinotecan have been studied to modulate the anti-cancer activity of 5-FU. For metastatic CRC, not all patients benefit from chemotherapy and are exposed to toxic or lethal effects unnecessarily in many cases (Gray *et al.* 2007).

The presence of drug resistant micrometastic tumour cells are also likely to reduce the effectiveness of adjuvant chemotherapy following surgery (Longley *et al.* 2006). Characterization of the biological factors that correlate with response to 5-FU-based chemotherapy is crucial in defining patients who are most likely to benefit from the treatment. Molecular markers such as enzymes involved in 5-FU metabolism (Soong *et al.* 2008), CpG island methylator phenotype (CIMP) status (Van Rijnsoever *et al.* 2003) and microsatellite instability (MSI) (Popat and Houlston 2005; Sinicrope *et al.* 2006) have been associated with patient outcomes from chemotherapy in CRC. The validation of predictive markers not only helps to predict tumour and patient response to chemotherapy, but also permits selection of the best combined treatment for an individual patient (Longley *et al.* 2006).

4.3 Adaptive Immunity in Cancer

The definition and key concepts of 'immune surveillance' were first established by Burnet and Thomas in 1960s (Burnet 1967). Burnet believed that tumour cell-specific neo-antigens could provoke an effective immunologic reaction that would eliminate developing cancers (Burnet 1957; Burnet 1964; Burnet 1971). Alternatively, Thomas suggested that complex long-lived organism must possess a mechanism to protect against neoplastic disease similar to those involved in homograft rejection (Thomas

1959). However, the cancer immunosurveillance hypothesis only started to gain recognition after the ideas of Burnet and Thomas were supported by the functional demonstration of tumour-specific antigens in mice (Old and Boyse 1964).

Accumulating reports have begun to elucidate the cellular basis of cancer immunosurveillance and demonstrate that lymphocytes of both innate and adaptive immune compartments prevent tumour development (Dunn *et al.* 2004). Studying activation of dentritic cells (DCs) by endogenous signals (Matzinger 1994; Gallucci *et al.* 1999; Gallucci and Matzinger 2001) and their interaction with cytotoxic T lymphocytes (CTLs) (Ridge *et al.* 1996; Bennett *et al.* 1998; Schoenberger *et al.* 1998), Smyth *et al*. proposed a model of adaptive immune response to tumour-derived tumour-associated-antigens (TAA) in melanoma (Smyth *et al.* 2001). 'Danger' signals such as cytokines or heat shock proteins (HSPs) (Gallucci *et al.* 1999; Gallucci and Matzinger 2001) that are released by cells undergoing damage and necrotic death cause activation of antigen-presenting DCs. Once activated, DCs initiate and control an adaptive immune response toward tumour-associated-antigens (TAAs). TAAs, processed and presented by major histocompatibility class (MHC) class I and class II molecules on a single DC, then enable priming and activation of both CD4+ T cell and CD8+ T cells (Ridge *et al.* 1996; Bennett *et al.* 1998; Schoenberger *et al.* 1998). Ultimately, activated antigen-specific CD8+ T cells differentiate into CTLs and destroy tumour cells (Figure 1).

How chemotherapy could modulate the host immune system in cancer patients is still not fully elucidated. Although the actions of different chemotherapeutic drugs vary, their effects on cancer cells are similar (Lake and van der Most 2006). Animal experiments conducted by Casares *et al.* showed that after the lethal effects of

chemotherapy, the apoptotic cancer cells become more 'immunogenic', and can excite the immune system (Casares *et al.* 2005). This event stimulates a second wave of cell killing via the adaptive immune response that can further improve the outcome from chemotherapy (Figure 2) (Lake and van der Most 2006). In contrast, Shankaran *et al.* (Shankaran *et al.* 2001) has shown that the immune system may also promote the emergence of primary tumours with reduced immunogenicity that are capable of escaping immune recognition and destruction. This is known as immunoediting. Tumour immunity, which initially provides host protection from cancer may ultimately drive the generation of tumours better suited to survive in an immunologically intact environment (Dunn *et al.* 2004).

Undoubtedly, improved understanding of the immunobiology of cancer immunosurveillance and immunoediting will help in developing immunotherapy to control and/or eradicate neoplastic disease in cancer patients (Dunn *et al.* 2004).

4.4 Tumour Infiltrating Lymphocytes (TILS) And Subtypes

4.4.1 Phenotypic and functional characteristics of TILS, Regulatory T cells (Tregs) and cytotoxic T cells (CTL)

Cancers are commonly infiltrated by immune cells along their invasive margin. The most frequent cell types are T and B lymphocytes and a minority are dendritic cells, natural killer cells and macrophages (Ohtani 2007). The anti-tumour activity of these cells has been well established in pre-clinical models, making it highly plausible that the immune cell density, type and function could affect the clinical phenotype of tumours.

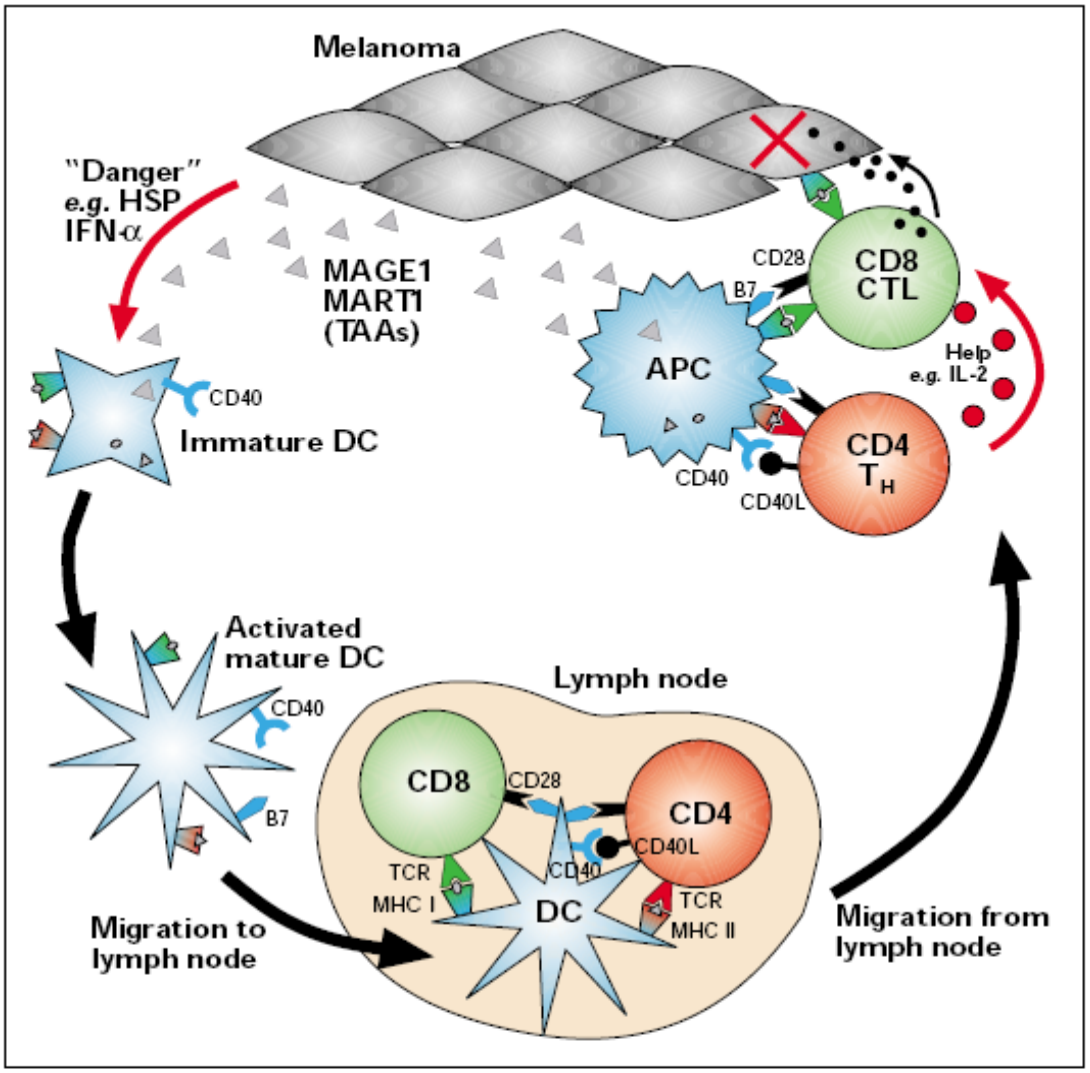

Figure 1. The adaptive immune response to tumour-derived TAAs such as MAGE1 and MART1 in melanoma.

One of the earliest steps toward mounting an adaptive response to TAAs is the capture and presentation of these molecules by professional APCs such as DCs. Additionally, to present costimulatory molecules, such as B7, to T lymphocytes, APCs must be activated by danger signals. In conjuction with costimulation (for example, B7-CD28 interaction), activated APCs carry TAAs to lymph nodes where the TAA-derived peptides are presented via MHC class II molecules to CD4+ and MHC class I molecule to CD8+ T lymphocytes. Stimulated CD4+ T cells subsequently express CD40L, which, in turn, further stimulates CD40-expressing APCs. B lymphocytes (not shown) are also likely to be involved in this immune response. TAA-specific lymphocytes develop into activated effector cells—CD8+ CTLs and CD4+T helper cells—with the ability to migrate into the tissue to mount an attack against the developing melanoma. This is likely to involve both CD4+, CD8+ T cells and local APCs (possibly B cells, macrophages and DCs). APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HSP, heat shock protein; T_H, T helper lymphocyte. (Adapted from Smyth *et al.* 2001)

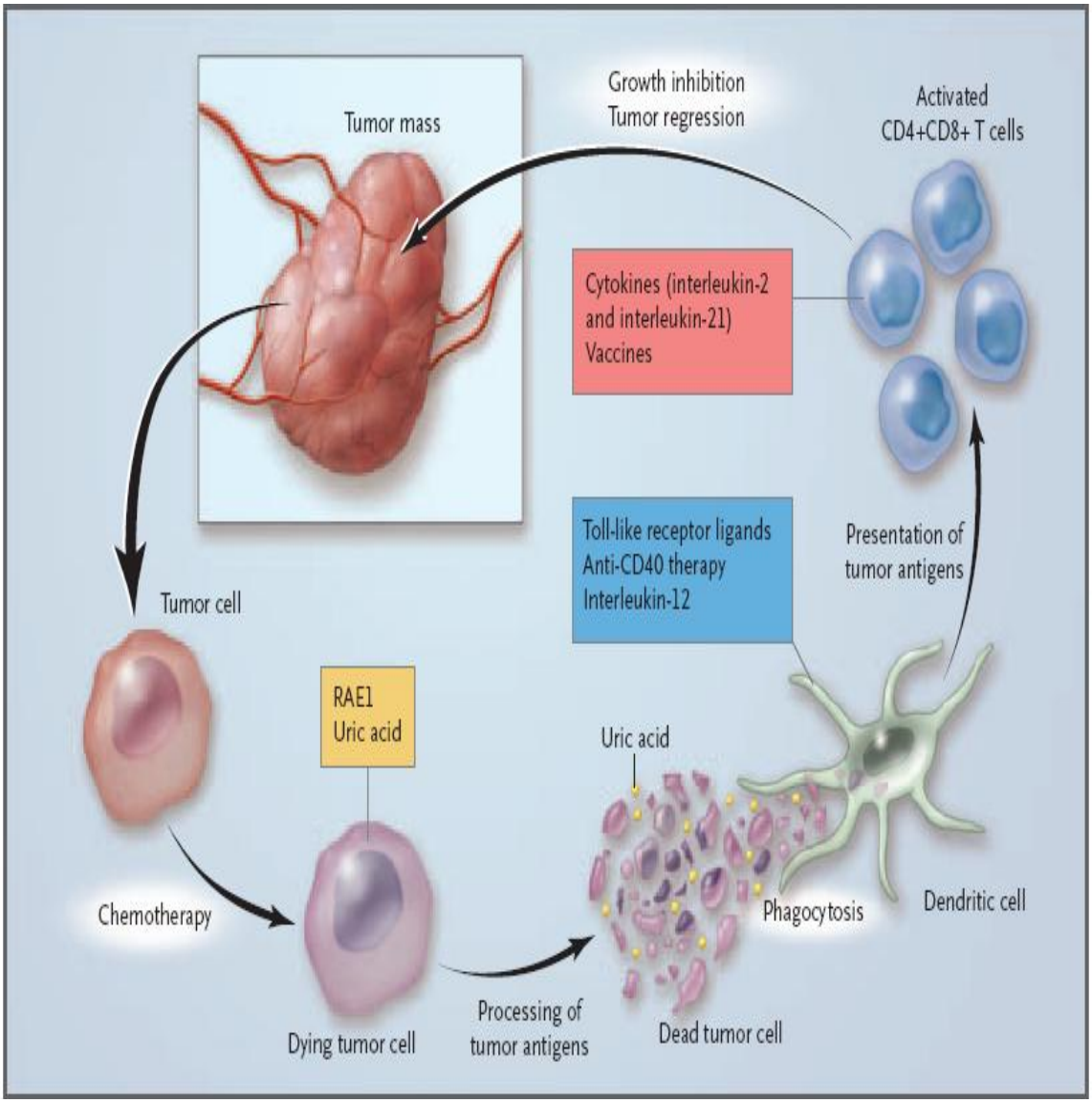

Figure 2. Harnessing the immune system to kill cancer cells.

Cancer cells that die after treatment with cytotoxic agents can be processed by dendritic cells for presentation to CD4+CD8+ T cells. Some drugs initiate a program of targeting and killing cells that amplifies this process. Dying cells then express molecules that serve as danger signals (yellow box) that activate dendritic cells to prevent cancer antigens in an inflammatory context. In response, dendritic cells up-regulate costimulatory molecules and secrete cytokines such as interleukin-12. Immunotherapies that target dendritic cells (blue box) may induce an effective immune response even if danger signals are not expressed by dying tumour cells. Additional immunotherapies directed at T cells (red box) may also promote antitumour immunity. (Adapted from Lake and van der Most 2006)

The major T lymphocyte subtypes within TILS can be identified by distinctive cellular markers. CD3 total T lymphocytes play a central role in cell-mediated immunity and they can be classified into several different subsets of T cells with distinct functions. The most commonly discriminated types include CD8+ cytotoxic cells, CD4+ effector cells and CD45RO+ antigen sensitized memory T cells. Also included are CD4+/CD25+/FOXP3+ and CD8+/CD25+/FOXP3+ regulatory cells, referred to as T4regs and T8regs respectively.

Cytotoxic T cells are also known as killer T cells because of their capability to induce the death of infected, damaged, or dysfunctional cells. In general, cytotoxic T cells express the glycoprotein CD8, and can recognize specific antigen peptides bound to class I major histocompatibility complex (MHC) molecules. The activation of CTL requires at least two simultaneous interactions between receptors expressed on the surface of T cells and ligands on the surface of antigen-presenting cells (APC). The first signal is provided by the interaction of the T cell receptor of CTL with a specific antigen that is presented on the surface of the APC. The second signal is a co-stimulatory signal which is mediated by interactions between CD28 of CTL and costimulators (such as CD80 or CD86) which are expressed on the APC. Once CTLs are activated, they are highly cytolytic and able to induce death through releasing perforin and enzyme proteases (granzymes) or Fas-FasL interaction.

Regulatory T cells were first discovered in 1970 (Gershon and Kondo 1970). Since then, the physiological and pathological role of Tregs in mouse and man have been widely studied. Some Tregs derive from the thymus and are known as “natural Tregs” (nTregs) (Sakaguchi *et al.* 1995), whereas other T cells that have been induced to become Tregs (iTregs) in peripheral lymphoid organs (Bluestone and Abbas 2003).

Treg cells are known as a minor population of CD4+ T cells expressing CD25 and the alpha subunit of interleukin 2 (IL-2) receptor (Sakaguchi *et al.* 1995). However, these markers were found to also be abundantly expressed on activated T cells. In 2003, the fork head box transcription factor FOXP3 was identified as a bona fide marker of Tregs and its expression is crucial for regulatory activity (Fontenot *et al.* 2003). In addition to CD4 Treg cells, regulatory CD8 T cells (CD8 Treg) have also been identified and suggested to play an important role in immune tolerance (Ciubotariu *et al.* 1998; Liu *et al.* 1998; Kumar and Sercarz 2001; Field *et al.* 2003).

Treg cells can inhibit the function of antigen-presenting cells or other cells of the innate immune system through four mechanisms: (i) secretion of suppressor cytokines (eg. IL10, TGF-b, IL-35 etc.) that can directly inhibit the function of responder T cells and myeloid cells; (ii) induction of high expression of CD25 on the activated Treg cell surface, which has the capacity to compete with effector T cells for IL-2, leading to Bim-mediated effector T cells apoptosis; (iii) expression of galectin-1 on activated Treg cells, which can interact with effector T cells and lead to their cell cycle arrest; (iv) direct killing of effector cells in a CD8+ cytotoxic cell-like manner by activated FOXP3+Treg cells (Shevach 2009).

4.4.2 Prognostic Significance

High levels of TILS are associated with favourable prognosis in several cancer types including breast (Marrogi *et al.* 1997), ovarian (Zhang *et al.* 2003), mesothelial (Leigh and Webster 1982) and colorectal cancer (Ropponen *et al.* 1997; Naito *et al.* 1998; Guidoboni *et al.* 2001; Menon *et al.* 2004; Prall *et al.* 2004).

CD8+ T cells or the activated CTLs have been shown as a predictor for improved survival in breast (Guo *et al.* 2008), gastric (Lee *et al.* 2008), liver (Gao *et al.* 2007),

lung (Kawai *et al.* 2008), melanoma (van Houdt *et al.* 2008) and ovarian cancer (Sato *et al.* 2005; Callahan *et al.* 2008; Leffers *et al.* 2009). In CRC, infiltrating CD8+ cells in the cancer nest have been reported to be significantly associated with better survival and possess higher cytotoxic and proliferative activity than those in the stroma and invasive margins (Naito *et al.* 1998). CD8+ T cells have also been associated with microsatellite instability (MSI) in CRC tumours (Guidoboni *et al.* 2001; Prall *et al.* 2004; Salama *et al.* 2009). Numerous mutations that occur in the MSI tumours lead to production of potentially immunogenic epitopes and subsequently induce CTL priming and cytotoxic activity.

High infiltration of Treg cells has been associated with poor prognosis or high cancer recurrence in various cancer types such as melanoma (Miracco *et al.* 2007), breast (Bates *et al.* 2006), ovarian (Curiel *et al.* 2004; Sato *et al.* 2005), hepatocellular (Gao *et al.* 2007; Kobayashi *et al.* 2007) and pancreatic cancers (Hiraoka *et al.* 2006). In contrast, a better survival for CRC patients with high intra-tumoural Treg has been reported (Salama *et al.* 2009; Correale *et al.* 2010; Frey *et al.* 2010). This suggests that Treg cells may act as homeostatic controllers of a robust immune response instead of immunosuppressors in CRC (Matera *et al.* 2010).

4.4.3 Predictive Significance

Chemotherapy can induce a second round of massive tumour cell death by increasing antigen release and up-regulating immunogenic surface molecules on apoptotic tumour cells (Lake and Robinson 2005). High density of TILS was found to predict survival benefit from 5-fluorouracil-based (5-FU) adjuvant chemotherapy in stage III colon cancer patients (Morris *et al.* 2008). The percentage of intra-tumoural lymphocytes was also shown to be a significant parameter for complete pathologic

response in breast tumours treated with anthracycline-based neoadjuvant therapy (Denkert *et al.* 2010). These clinical observations support the notion that conventional cytotoxic chemotherapy can be a potent activator of pre-existing antitumour immune responses in cancer (Lake and van der Most 2006).

In the phase II trial of a chemoimmunotherapy regimen, which combined gemcitabine, oxaliplatin, levofolinic acid, 5-FU, granuclocyte macrophage colony-stimulating factor and interleukin 2 (GOLFIG-1), patients who received the treatment were found to have increased level of CTL in their peripheral blood (Correale *et al.* 2008). In a murine study, treatment with gemcitabine results in increased antigen cross-presentation and priming of tumour-specific CD8 cells.

Tregs have been found to be sensitive to chemotherapy. Low-dose oral cyclophosphamide in patients with advanced breast cancer selectively depleted the Treg subset and enhanced the cytotoxic capacity of T and natural killer cells (Diaz-Montero *et al.* 2009). Similarly, circulating Tregs were greatly reduced in breast cancer and CRC patients who received anthracycline-based regimen (Ladoire *et al.* 2008) and GOLFIG (Correale *et al.* 2008) respectively.

4.5 Circulating immune cells

The immune system is made up of a network of cells, tissues and organs that work together to protect the body. Hence, host response in tumours may also be reflected in other counterparts such as peripheral blood, peritoneal cavity, bone marrow or lymph node. Higher frequencies of T4reg (Clarke *et al.* 2006; Ling *et al.* 2007; Ahmadzadeh *et al.* 2008) and T8reg cells (CD8+FoxP3+) (Chaput *et al.* 2009) were observed in peripheral blood of cancer patients compared to healthy individuals. This has

suggested that the activation of immune response may possibly be systemic than localized in tumours.

Peripheral immune cells were also found to be associated with clinical response to chemotherapy in CRC patients (Lissoni *et al.* 2006; Correale *et al.* 2008). Lissoni *et al*. reported an increase in the total peripheral blood lymphocyte count in patients who showed clinical response to chemotherapy with 5-FU and oxaliplatin, but a decrease in those who showed stable or progressive disease (Lissoni *et al.* 2006). However, studies on the prognostic and predictive values of peripheral blood cells are still preliminary.

4.6 Single Nucleotide Polymorphism (SNP)

A single nucleotide polymorphism (SNP) is defined as a change in a single nucleotide when alleles are compared. It occurs every ~1330 bases in the human genome (Lewin 2004). SNP can be found within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. When SNPs occur in the coding region of a gene, they may produce the same polypeptide sequence (also known as synonymous change) or different polypeptide sequence (non synonymous change). A non-synonymous change may be either missense or nonsense that leads to different amino acid or premature stop codon respectively. For SNPs that are not in the protein-coding regions, they may still contribute to changes in gene splicing, transcription binding or affect the transcription of non-coding RNA.

The immune system has the most complex task of responding to evolving environmental components that enter the organism through different routes in the form of pathogens (Jin and Wang 2003). Under this pressure, immune related genes

such as major histocompatibility complex (MHC), cytokines and toll-like receptors have been found to be polymorphic (Jin and Wang 2003; El-Omar *et al.* 2008). Inevitably, polymorphisms in immune-related genes can be associated with risk of infectious diseases (Kang and Chae 2001), allergy (Yang *et al.* 2004), cardiovascular disease (Girnita *et al.* 2009) and cancer (El-Omar *et al.* 2000; Sun *et al.* 2005; Hold *et al.* 2007).

A variety of commercial platforms are developed to tailor for semi-automated or fully automated SNP genotype analysis (Table 1) (Ding and Jin 2009). Basically, the chemistry for SNP genotyping can be classified to two types: non-enzymatic differential hybridization and enzymatic reaction. Differential hybridization relies on different melting temperatures for matched and mismatched oligonucleotides binding to the target DNA sequences. Enzymatic reaction methods, two types of assays using incorporation of fluorescent nucleotides, and extension of product molecular weight have been developed. In general, differential hybridization based platforms rely entirely on hybridization thermodynamic differences between matched and mismatched pairing of probes and targets. Therefore, the probes must be effective in differentiating matched and mismatched targets. For enzymatic selectivity based platforms are less dependent on SNP local sequences, provide less background noise and allow for more SNPs detection (Ding and Jin 2009).

Table 1. Comparison of high throughput genotyping platforms.

Platform	Provider	Chemistry	Detection	Number of SNPs	Number of samples	Note	URL
iSelect BeadArray	Illumina	Single base extension or allele-specific primer extension	Fluorescence	Up to 60,800	12	Design and delivery take at least 3 months	http://www.illumina.com/pages.ilmn?ID=158
GeneChip custom SNP kits	Affymetrix (ParAllele)	Molecular inversion probe (primer extension and ligation)	Fluorescence	3,000, 5,000, or 10,000	1	Design and delivery take at least 3 months	http://www.affymetrix.com/products/reagents/specific/custom_snp.affx
MassArray	Sequenom	Single base extension	Molecular weight (Mass Spec)	Up to 40	384		http://www.sequenom.com/Genetic-Analysis/Applications/iPLEX-Genotyping/iPLEX-Overview.aspx
SNPlex	Applied Biosystems	Allele-specific ligation	Probe size (capillary electrophoresis)	Up to 48	96 or 384		https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=600763
SNPstream	Beckman Coulter (Orchid)	Single base extension	Fluorescence	12 or 48	384	Call rate at higher plex level may be low	http://www.beckmancoulter.com/products/instrument/geneticanalysis/ceq/genomelab_snpstream_dcr.asp
TaqMan OpenArray	Applied Biosystems and BioTrove	TaqMan	Fluorescence	64[a]	48		http://www.biotrove.com/products/open_array/snp/index.asp

SNP single nucleotide polymorphism
[a]Not true multiplexing, 64 uniplex TaqMan SNP assays in 64 different nano holes.

4.7 Scope of Study

The overall aims of the study were to understand the clinical relevance of adaptive immunity in CRC tumours. We first evaluated the prognostic (survival) and predictive (response to chemotherapy) value of tumour-infiltrating lymphocytes (TILS) subtypes in CRC tumours. Secondly, we examined the correlation between the circulating lymphocytes (blood) and TILS of CRC patients and screened for SNPs of T cell markers that may significantly associate with T cell densities. The specific aims were:

Specific Aim 1: To characterize both the prognostic and predictive values of various tumour-infiltrating T-lymphocyte subtypes in CRC patients receiving 5-FU-based chemotherapy.

Hypothesis: CRC patients with low Tregs densities and high cytotoxic T cell densities will be more responsive to chemotherapy.

Specific Aim 2: To assess the correlation between the densities of T cell subtypes in the peripheral blood and primary tumours of CRC patients.

Hypothesis: Individuals with high levels of circulating T cells are the individuals that exhibit TILS.

Specific Aim 3: To characterize the correlations between SNPs in *CD8a*, *GZMB* and *FOXP3* genes in CRC patients and respective gene expression and T cell densities in peripheral blood and tumours.

Hypothesis: TILS and high circulating immune cells could be a manifestation of genetic differences that encode a heightened immune system.

5 MATERIALS AND METHODS

5.1 Workflow

A workflow of the study methods is given below in Figure 3.

5.2 Cases and Tissue Arrays

Tissue microarrays (TMA) containing cores from 439 routinely fixed and processed primary tumour specimens were obtained from consecutive patients who underwent surgical resection for histologically proven CRC at the National University Hospital of Singapore between 1990 and 1999. Details of the TMA construction and relevant patient data including the use of adjuvant chemotherapy with 5-FU-based regimens have been described previously (Ong *et al.* 2010). The available clinical and pathological information included gender, age, tumour size, tumour stage (AJCC), histological grade, vascular invasion, perineural invasion, and lymphatic invasion. Survival time was measured from the initial date of diagnosis until the date of cancer-specific death, or censored at the time of last follow-up if the patient was still alive, lost of follow-up or had died of other causes. Some 99 cases were alive, 212 died from CRC, 88 died from other causes and 40 were lost of follow-up. Patients were selected to receive adjuvant chemotherapy based on age, the presence of "poor" prognosis features and patient/doctor preference in the course of routine clinical practice. Cases treated with chemotherapy underwent intravenous injection with 5-fluorouracil (5-FU) using the Mayo regimen. One complete cycle of treatment involved dose administration ranging from 500 to 900 mg/m^2/day for 3 to 5 consecutive days. Each cycle was repeated monthly for 6 months or until progression

of disease, patient refusal or adverse reactions to the treatment.

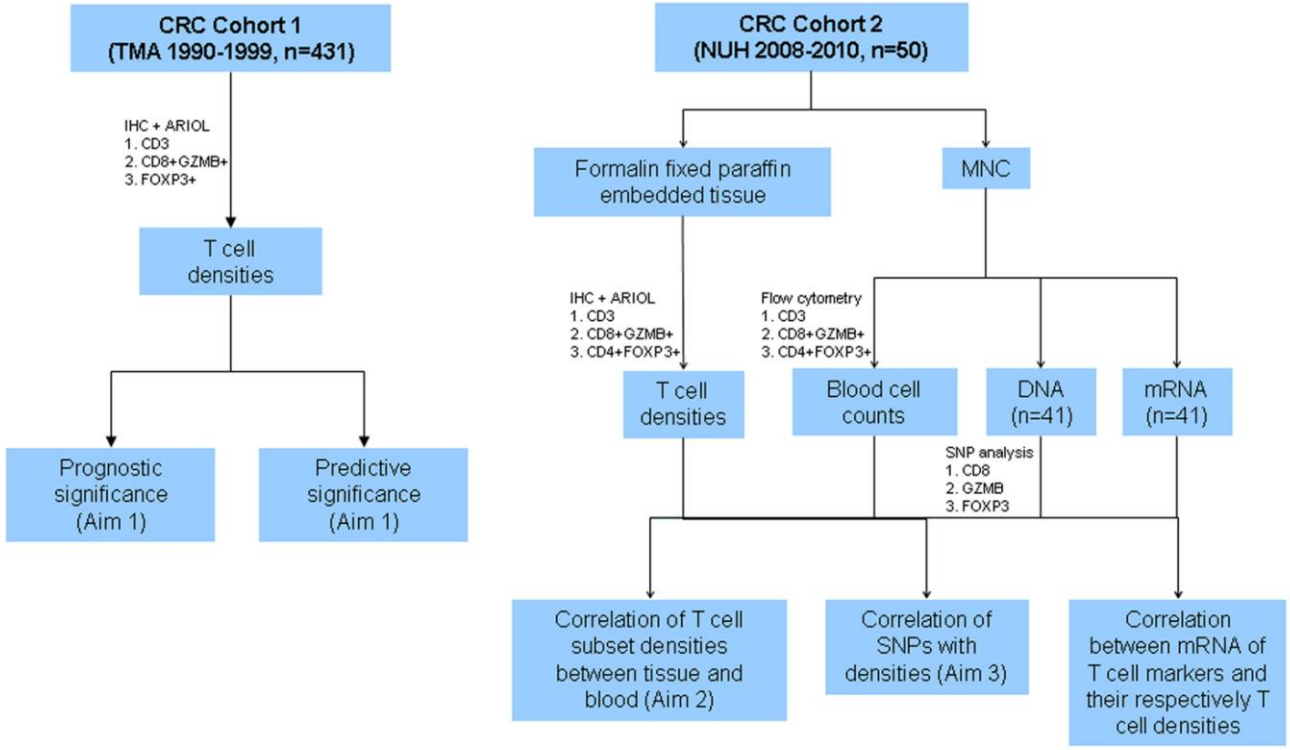

Figure 3. Overview of study workflow.

IHC, immunohistochemistry; Ariol, automated slide imaging platform; MNC, mononuclear cells.

To assess the correlations of the densities of T cell subtypes between the matched peripheral blood and primary tumours, fifty CRC patients were recruited from National University Hospital (NUH) Department of Surgery and Haematology Oncology between 2008 and 2010. Blood samples from these patients were drawn before surgery. None of the patients had pre-operation radiotherapy or chemotherapy. A total of 7ml of peripheral blood was drawn into Vacutainer CPT tubes (Becton Dickinson, USA) and mononuclear cells (MNC) were collected after centrifugation at 2500 rpm for 20min. MNCs were washed once with 6ml Roswell Park Memorial Institute medium (RPMI) supplemented with 10% fetal bovine serum (FBS) before re-suspending in 2.4ml freezing medium (40% RPMI, 50% FBS, 10% DMSO). MNCs were kept in cryovials and stored in -80°C for 24 hr and thereafter liquid nitrogen for long-term storage. Corresponding patient paraffin embedded tumour samples containing >50% tumour cells were collected from NUH Department of Pathology. Sections of 4-µm thickness were used for immunohistochemical staining. The protocol for this study was approved by the Institutional Review Board of the National University of Singapore (NUS-IRB reference code: 09-429).

5.3 Immunohistochemistry

Consecutive TMA and full face paraffin sections of 4µm thickness were cut and placed onto silanated slides for immunohistochemical detection of various T-lymphocyte markers using a Bond-Max autostainer (Leica Biosystems, Newcastle, UK). For single staining of CD3 and FOXP3, heat-induced antigen retrieval with epitope retrieval ER1 solution (Leica Biosystems) was performed for 20 min prior to incubation with primary antibody. Slides were incubated with CD3 (Novocastra, Clone LN10, dilution 1:100) or FOXP3 (Abcam, clone 236/E7, dilution 1:250) at

room temperature (RT) for 15 min. After incubation, slides were washed three times with 1x Bond washing buffer (Leica Biosystems) and incubated with secondary antibody (Bond Polymer Refine kit, Leica Biosystems) for 8 min at room temperature. Chromogenic detection was achieved by incubation with 3,30-diaminobenzidine (DAB) for 10 min, followed by 5 min incubation with Bond DAB enhancer.

For the double staining of GZMB/CD8 and CD4/FOXP3, heat-induced antigen retrieval with epitope retrieval ER2 solution (Leica Biosystems) was performed for 20 min prior to incubation with primary antibody. Anti-GZMB (Novocastra, clone 11F1, dilution 1:40) or anti-FOXP3 (Abcam, clone 236/E7, dilution 1:250) antibodies were incubated for 30 min or 15min respectively at RT and then detected using the DAB chromogenic method described above. The slides were subsequently incubated with anti-CD8 antibody (DakoCytomation, clone 144B, dilution 1:600) or anti-CD4 antibody (Novocastra, clone 4B12, dilution 1:50) and secondary antibody from the Bond Polymer Alkaline Phosphatase Red detection kit (Leica Biosystems) to give a distinct red stain with DAB staining. Negative controls comprised the omission of primary antibody, while internal positive controls for the immune cell markers were provided by lymphoid follicles present in adjacent normal colonic mucosa.

All slides were counterstained with haematoxylin and dehydrated through ascending ethanol to Histo-Clear solution (National Diagnostics, Atlanta, GA) before mounting. Slides were scanned with a high resolution scanner (Ariol SL-50, Applied Imaging, San Jose, CA) at 100X and 400x magnifications. For TMA, the number of positively stained lymphocytes in the intraepithelial and stromal areas was counted by visual observation for each core and the density of lymphocytes for each tumour was then expressed as cells per square millimeter as described previously (15). For full face

paraffin sections, five regions (1mm^2 each) with the highest infiltration of CD3 positive lymphocytes were marked by pathologist. The positively stained cells were counted and the cell densities were determined by taking the average of the 5 regions. Scoring was performed by two observers (CPY and BT) without prior knowledge of patient characteristics. Inter-observer agreement was greater than 0.837 for the stains by the Kappa test.

5.4 Microsatellite Instability Analysis

Tumour blocks corresponding to 278 cases in the tissue array were retrieved from Pathology archives. Three 8μm sections were cut from each block, and DNA was extracted using the Gentra Purgene Tissue kit (Qiagen, Hilden, Germany). Microsatellite instability was determined by analysis of 5 mononucleotide repeats, including BAT-25, BAT-26, NR21, NR22, NR24 and NR27, as reported by Buhard *et al*. (Buhard *et al.* 2006). Tumours were defined as MSI when ≥3 markers showed instability.

5.5 Flow Cytometry

Frozen MNCs were quickly thawed in 37°C water bath. Cells were washed with 5ml RPMI supplemented by 10% Fetal Bovine Serum (FBS) followed by 5ml of staining buffer (phosphate-buffered saline [PBS] with 3% FBS). Cells were then spun at 1200rpm for 5min. The pellets were re-suspended in 100μl of cold staining buffer and stained with antibodies against surface antigens (CD3 and CD4 or CD8) for 30min in the dark at 4°C. Cells were subsequently washed in staining buffer twice prior to fixation. For FOXP3 and GZMB, cells were permeabilized using the Foxp3 Staining Buffer Set (eBioscience, San Diego, CA) according to the manufacturer's instructions. Negative controls comprised the omission of antibodies against surface

markers and allophycocyanin-conjugated isotype control antibody of anti-FOXP3 (eBioscience) or anti-granzyme B (Invitrogen) only. All the dilutions of antibodies were according to the manufacturers' recommendation.

Stained cells were analyzed on FACS LSRII flow cytometry instrument (BD Bioscience, San Jose, CA) and data were analyzed using FlowJo software (TreeStar, Ashland, OR). Quadrants were set based on negative controls. The information for the antibodies mentioned above and their dilutions used are listed in Table 2. The cell densities were expressed as percentage of positive cells of total CD3+ T cells.

Table 2. Antibodies and dilutions used for flow cytometry.

Antibody	Manufacturer	Clone	Fluorochrome	Vol. used (μl)
Treg markers				
CD3	BD	SK7	APC-Cy7	20
CD4	BD	RPA-T4	FITC	20
FOXP3	eBioscience	PCH101	APC	5
Cytotoxic T cell markers				
CD3	BD	UCHT1	FITC	20
CD8	eBioscience	RPA-T8	PE-Cy7	5
Granzyme B	Invitrogen	GB12	APC	5

5.6 DNA and RNA Extraction

Frozen MNCs were quickly thawed in a 37°C water bath. Cells were washed with 5ml of PBS and centrifuged at 5,000 rpm for 2 min at 4°C. Trizol (Invitrogen, Carlsbad, CA) was used to extract total RNA and DNA from the cell pellets according to manufacturer's instructions. The yields and purity of RNA and DNA were determined spectrophotometrically at 260nm and 280nm using a Nanodrop (Thermo Scientific, Wilmington, DE).

5.7 Gene expression analysis

A total of 0.5µg of total RNA was treated with (RNAse-free) DNAse I (New England Biolabs, Ipswich, MA) for 10min at 37°C. DNAse I activity was inactivated by adding EDTA and heating at 75°C for 10min. The integrity of RNA was determined by the ratio of 28S and 18S values using Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the RNA 6000 Nano kit. A total of 250ng of DNAse-I treated RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA). The reaction mixtures were incubated at 25°C for 10 min, then 37°C for 120 min and 95°C for 5 min.

The expression of *CD8a* (Hs00233520), *GZMB* (Hs01554355), *FOXP3* (Hs01085834) and *ACTB* (Hs99999903) was analyzed by real time PCR (QPCR) using TaqMan® custom gene expression assays (Applied Biosystems). All reactions were performed in 20 µL reactions, in duplicate within the same PCR run. Negative controls consisting of the addition of water for each gene target analyzed were included in each PCR. For each well 1µl of cDNA (equivalent to 12.5ng of RNA) from each sample was added to 20µl of PCR reaction mix consisting of 10× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster city, CA) and 1µl gene expression assay primer-probe mix. PCR was initiated with a 10 minute incubation at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, in accordance with the manufacturer's recommendations. Real-time PCR and relative quantification were performed using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The expression values of target gene were normalized to *ACTB* levels using SDS 2.2.1 software (Applied Biosystems).

5.8 Genotyping

SNP genotyping in clinical samples were carried out using the MassArray iPLEX genotyping platform (Sequenom, San Diego, CA) according to manufacturer's instructions. SNPs, consisting of 10 in *CD8a*, 22 of *GZMB* and 7 of *FOXP3* (Table 8) with a minor allele frequency (MAF)≥1% were selected from the dbSNP database. Three assays comprising 19, 17 and 3 multiplex PCRs respectively were designed using both the MassArray Assay Design 4.0 and MassArray Online Design Tools (Sequenom). PCR amplification was carried out using 20ng of DNA in a 5µl reaction that contained 0.5U Taq polymerase, 10X PCR buffer, 4mM MgCl2, 500µM dNTPs and 0.1µM of primers. The following program was used for PCR amplification: 95^{o}C for 2 minutes, followed by 45 cycles of 95^{o}C for 30 seconds, 56^{o}C for 30 seconds, 72^{o}C for 1 minute and a final extension step of 72^{o}C for 5 minutes. Unincorporated dNTPs were removed using 0.3U shrimp alkaline phosphatase dissolved in the SAP buffer provided. Single-base extension was carried out in a 9µl reaction that contained iPLEX GOLD buffer, iPLEX termination mix, iPLEX extend primer mix and iPLEX enzyme. The reactions were performed using the following two cycling loop programs: initial denaturation was for 30s at 94°C followed by five cycles of 5s at 94°C, 5s at 52°C and 5s at 80°C. An additional 40 annealing and extension cycles were then looped back to 5s at 52°C and 5s at 80°C. The final extension was carried out at 72°C for 3min, before cooling to 4°C. Reactions were desalted using 6 mg clean resin and diluted with 16µl water. 10nl of each reaction was spotted onto the 384-spot SpectroChipII using the Nanodispenser. This was followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis using the MassARRAY Compact system. Spectra analysis and genotype calls were

generated using the TYPER 3.3 software (Sequenom).

5.9 Statistical Analysis

T-lymphocyte densities were classified as high or low relative to the median value. Chi-square analysis was used to examine for possible non-random associations between T-lymphocyte densities and clinicopathological features. Univariate Cox proportional hazards analysis was used to determine the prognostic significance of various clinicopathological features and T-lymphocyte subtype densities. Multivariate analysis was performed by Cox regression analysis (Method = "Enter") using the factors identified to be significant in univariate analysis. Predictive significance was determined by comparing the survival of patient groups treated with or without 5-FU chemotherapy in respective patient subgroups of interest. Differences in hazard ratios were assessed using the *hr.comp* function under the *survcomp* library in R (The R Foundation for Statistical Computing).

Associations between T cell densities in peripheral blood and tissue were determined by Pearson correlation coefficient. The same statistical method was also used to assess the correlation between the gene expression levels of the T cell markers and their corresponding cell densities in peripheral blood. Chi-square test with case weighting applied was applied to assess the difference between SNP distributions in the NUH cohort and other reported Asian cohorts. Chi-square test was also used to assess the associations between SNPs and the T cells densities in blood and tissue. T cell densities were dichotomized by median.

Significance was set at the 5% level and no corrections were made for multiple comparisons. Unless otherwise mentioned, statistical analyses were performed using

the SPSS package version 18.0 for Windows (SPSS, Chicago, IL).

6 PROGNOSTIC AND PREDICTIVE SIGNIFICANCE OF TILS SUBTYPE DENSITIES IN CRC

6.1 Introduction

Cancers are frequently infiltrated by immune cells along their invasive margin. The most frequent cell types are T and B lymphocytes, however the infiltrates also include dendritic cells, natural killer cells and macrophages (Ohtani 2007). The anti-tumour activity of tumour-infiltrating lymphocytes (TILS) has been well established in pre-clinical models, making it highly plausible that immune cell density, type and function could affect the clinical phenotype of tumours. Indeed, one of the most reproducible findings in prognostic studies has been that a high density of tumour-infiltrating lymphocytes (TILS) is associated with improved patient survival. In colorectal cancer (CRC) alone, at least 15 independent reports on the prognostic value of TILS have provided very consistent results (Broussard and Disis 2011). Similar findings have been reported for breast, prostate and small cell lung cancers. In many cases, the association has been independent of tumour staging, prompting some investigators to suggest this feature may even provide superior prognostic information to the TNM system in CRC (Pages *et al.* 2005; Galon *et al.* 2006; Mlecnik *et al.* 2011).

Nonetheless, an unresolved issue is whether an interaction between immune cells and chemotherapy could also be contributing to the better survival of patients with high levels of TILS. Cytotoxic treatment can destroy tumour cells by inducing necrosis, thus leading to the release of cytokines which can in turn activate cytotoxic T-lymphocytes at the tumour site (Lake and Robinson 2005). This event stimulates a

second wave of cell killing via the adaptive immune response that could further improve the outcome from chemotherapy. In support of this, we previously reported that the survival difference of patients treated with chemotherapy compared to those untreated was greater if the patients had a high compared to low TILS density (Morris *et al.* 2008). The density of immune cells in the primary tumour was also found to correlate with the response to chemotherapy in a study of metastatic CRC (Halama *et al.* 2009). In breast cancer, the presence of intra-tumoural lymphocytes was found to be a significant predictor for complete pathological response to neoadjuvant chemotherapy (Ladoire *et al.* 2008; Denkert *et al.* 2010).

TILS consist of many different subtypes, some of which can be readily identified by immunohistochemical staining for distinctive cellular markers. Anti-tumourigenic total, cytotoxic, activated cytotoxic and regulatory T-lymphocytes are identified by the expression of CD3, CD8, CD8/Granzyme B (GZMB) and FOXP3, respectively. The first three markers are linked to anti-tumour activities (Rosenberg 1996; Chowdhury and Lieberman 2008). FOXP3+ T-lymphocytes are postulated to suppress the activity of cytotoxic T-lymphocytes through direct cell-to-cell contact and through the release of cytokines, especially transforming growth factor-β (Clarke *et al.* 2006). With these contrasting activities, it is plausible that the observed prognostic and predictive values of TILS may differ according to the specific cell subtype. In the present study we examined these attributes for CD3+, CD8+, CD8+GranzymeB+ (GZMB+) and FOXP3+ cell densities in a cohort of 439 CRC patients for which extensive pathological and clinical information was available.

6.2 Results

6.2.1 Density and Cinicopathological Associations of T-lymphocyte subtypes in CRC

Representative images of single immunohistochemical stains for CD3 and FOXP3 and double stains for CD8/GZMB are shown in Figure 4. Activated cytotoxic T-lymphocytes were identified as cells that co-expressed CD8 at the cell surface and GZMB in the cytoplasm (Figure 4E). FOXP3 staining was observed exclusively in the nucleus of lymphocytes (Figure 4F). The distribution of cell densities for the different T-lymphocyte markers is provided in the Figure 5. The densities of all subtypes were significantly correlated to each other (Figure 6). Although FOXP3 staining is thought to represent T regulatory cells, positive correlations were seen with the densities of CD3+, CD8+ and CD8+GZMB+ cells. Associations between T-lymphocyte subtype densities and standard clinicopathological features for CRC are shown in Table 3. Early stage tumours (AJCC stage I and II) more often displayed high densities of the T-lymphocyte subtypes compared to late stage tumours ($P<0.01$ for each marker). With the exception of perforation and FOXP3+ cell density, no significant associations were seen between the other clinicopathological features and T-lymphocyte marker densities. Microsatellite instability (MSI) status was analyzable in 278 tumour samples, of which 31 (11%) had MSI. MSI tumours had higher densities of CD8+ ($P=0.04$) and CD8+GZMB+ T cells ($P<0.01$).

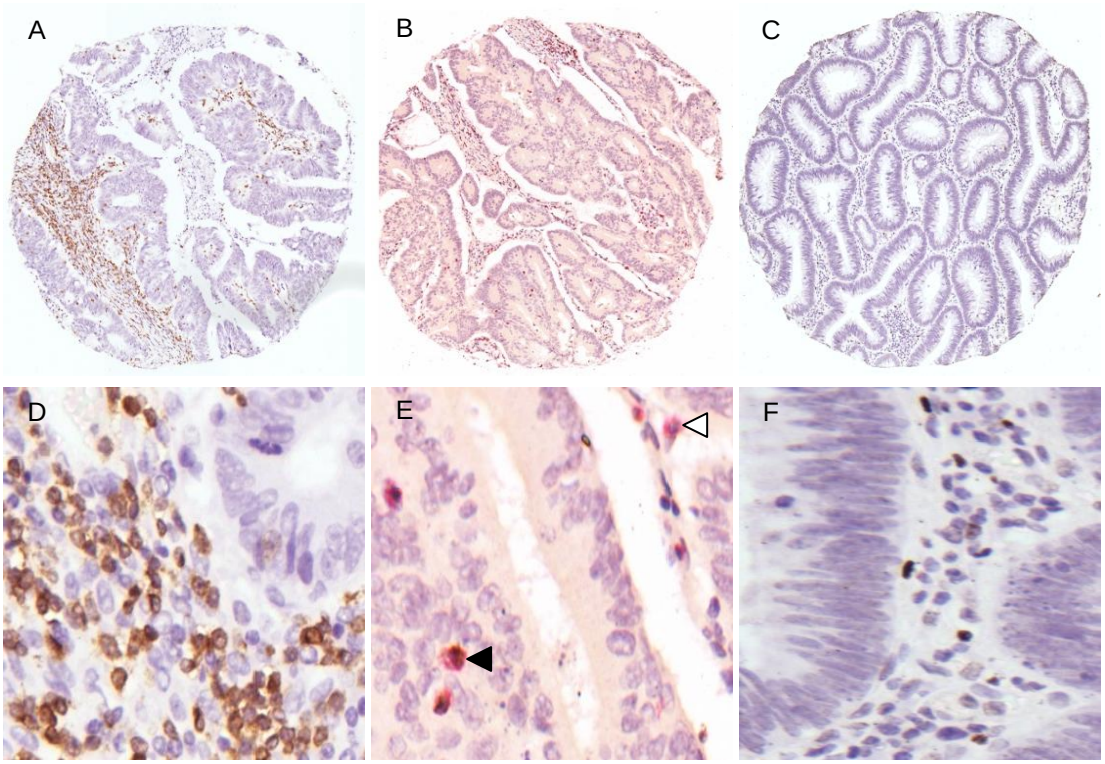

Figure 4. Immunohistochemical stains for CD3 (A, D), double staining of CD8 and GZMB (B, E), and FOXP3 (C, F).

In (E), the filled arrow (E) highlights an activated cytotoxic T lymphocyte with co-expression of CD8 (red) and GZMB (brown). The unfilled arrow highlights a cytotoxic T cell with no GZMB expression. Magnification for figure A-C: 100x; Magnification for figure D-F: 400x. Brown and red stains were achieved by incubation with DAB and alkaline phosphatase red respectively; and the nuclei were counterstained with haematoxylin.

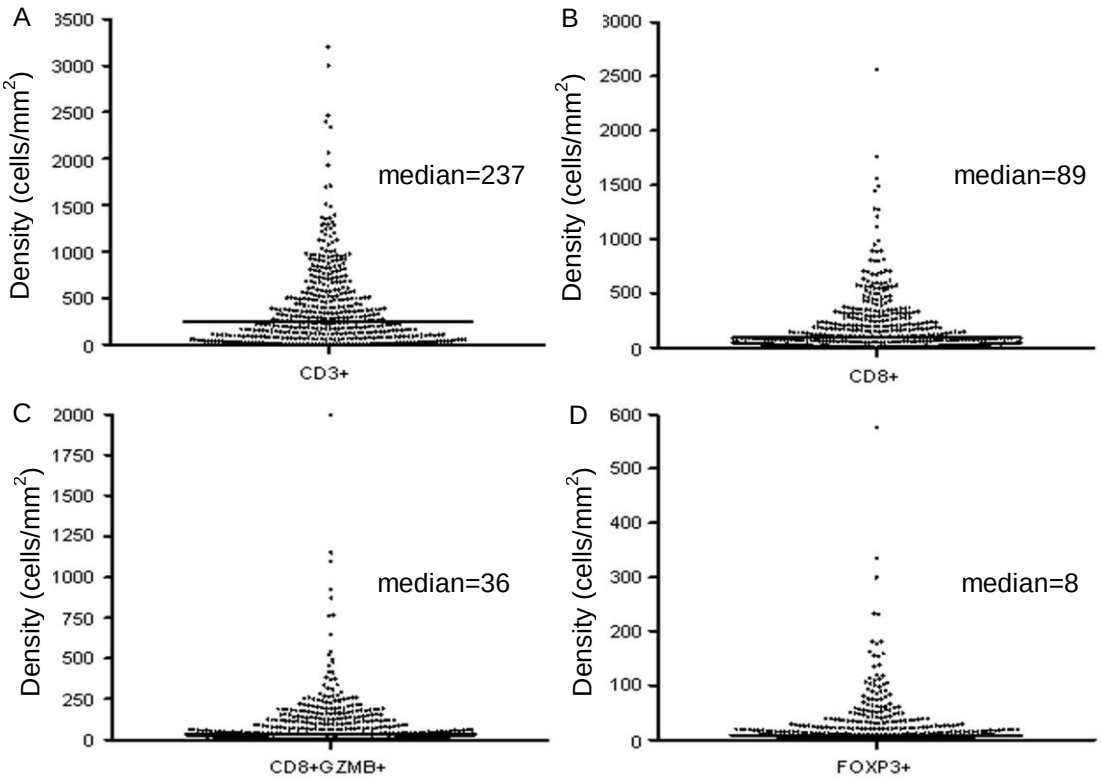

Figure 5. Density of respective T-lymphocyte subtypes detected by immunohistochemistry in CRC tissue samples.

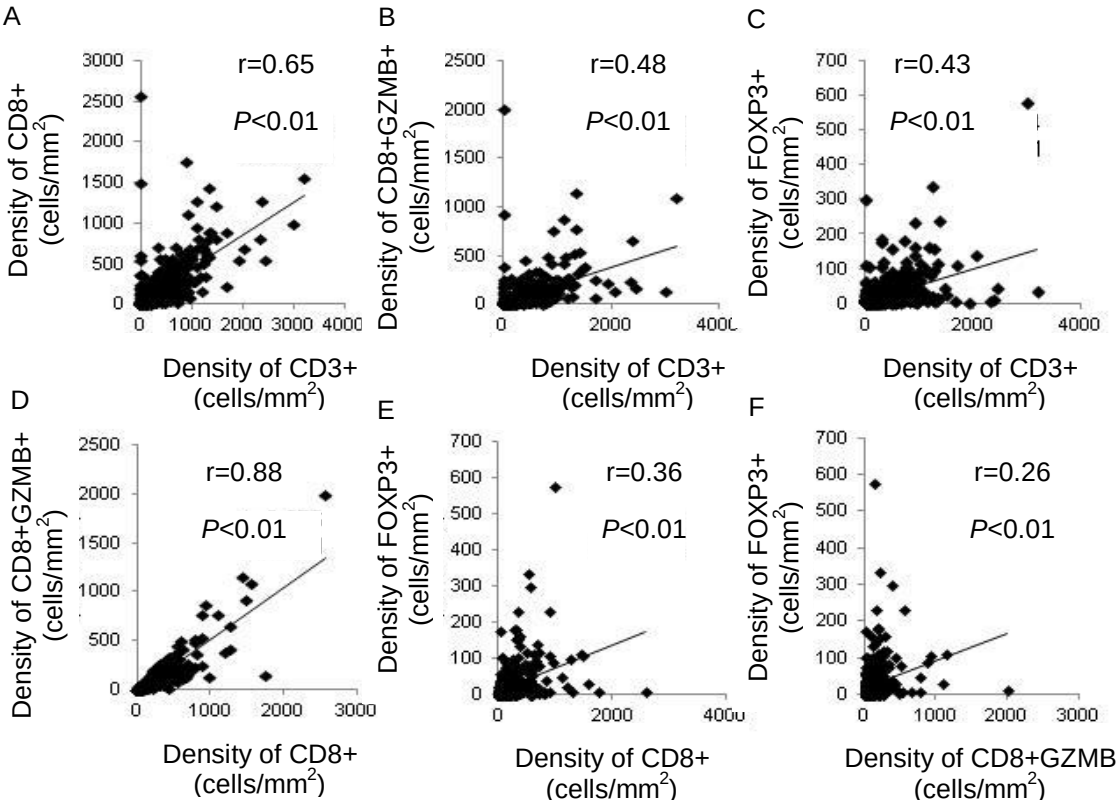

Figure 6. Correlation between T-lymphocyte subtype densities in CRC

Table 3. Associations between T cell subtype densities and clinicopathological parameters.

Feature	CD3+ High (%)	*P*	CD8+ High (%)	*P*	CD8+GZMB+ High (%)	*P*	FOXP3+ High (%)	*P*
Age								
<60 years (125)	64 (51.2)		66 (52.8)		66 (52.8)		59 (47.2)	
≥60 years (314)	156 (49.7)	0.83	156 (49.7)	0.60	160 (51.0)	0.75	166 (52.9)	0.29
Gender								
Male (213)	102 (47.9)		108 (50.7)		106 (49.8)		114 (53.5)	
Female (226)	118 (52.2)	0.39	114 (50.4)	0.52	120 (53.1)	0.51	111 (49.1)	0.39
Ethnicity								
Chinese (390)	194 (49.7)		199 (51.0)		202 (51.8)		202 (51.8)	
Non-Chinese (49)	26 (53.1)	0.76	23 (46.9)	0.65	24 (49.0)	0.76	23 (46.9)	0.55
Grade								
Well/Moderate (397)	198 (49.9)		196 (49.4)		202 (50.9)		204 (51.4)	
Poor (42)	22 (52.4)	0.87	26 (61.9)	0.15	24 (57.1)	0.52	21 (50.0)	0.87
Stage								
I & II (213)	124 (58.2)		123 (57.7)		124 (58.2)		124 (58.2)	
III & IV (226)	96 (42.5)	<0.01	99 (43.8)	<0.01	102 (45.1)	<0.01	101 (44.7)	<0.01
Site								
Proximal colon (161)	75 (46.6)		77 (47.8)		83 (51.6)		83 (51.6)	
Distal colon & rectum (278)	145 (52.2)	0.28	145 (52.2)	0.43	143 (51.4)	>0.99	142 (51.1)	>0.99
Tumour size								
<5cm (263)	134 (51.0)		130 (49.4)		131 (49.8)		144 (54.8)	
≥5cm (176)	86 (48.9)	0.70	92 (52.3)	0.63	95 (54.0)	0.44	81 (46.0)	0.08
Vascular invasion								
Absent (389)	195 (50.1)		196 (50.4)		198 (50.9)		200 (51.4)	
Present (50)	25 (50.0)	>0.99	26 (52.0)	0.88	28 (56.0)	0.55	25 (50.0)	0.88
Lymphatic invasion								
Absent (401)	199 (49.6)		202 (50.4)		205 (51.1)		205 (51.1)	
Present (38)	21 (55.3)	0.61	20 (52.6)	0.87	21 (55.3)	0.74	20 (52.6)	0.87
Perineural invasion								
Absent (418)	212 (50.7)		211 (50.5)		218 (52.2)		215 (51.4)	
Present (21)	8 (38.1)	0.27	11 (52.4)	>0.99	8 (38.1)	0.27	10 (47.6)	0.82
Perforation								
Absent (420)	212 (50.5)		214 (51.0)		218 (51.9)		220 (52.4)	
Present (19)	8 (42.1)	0.49	8 (42.1)	0.49	8 (42.1)	0.49	5 (26.3)	0.03
MSI								
Absent (247)	124 (50.2)		117 (47.4)		117 (47.4)		127 (51.4)	
Present (31)	16 (51.6)	>0.99	21 (67.7)	0.04	23 (74.2)	<0.01	20 (64.5)	0.19

6.2.2 Prognostic Significance of T-lymphocyte Subtype Density

Univariate survival associations for the standard clinicopathological features and of T-lymphocyte subtype densities are shown in Table 4. To eliminate the confounding influence of chemotherapy on survival outcome, only the patients treated by surgery alone (n=300) were evaluated. As expected, the factors of advanced tumour stage, poor histological grade and tumour invasion into vascular, lymphatic and perineural spaces were each associated with poor survival (all $P<0.01$). In agreement with many previous studies (Ropponen *et al.* 1997; Naito *et al.* 1998; Guidoboni *et al.* 2001; Chiba *et al.* 2004; Menon *et al.* 2004; Prall *et al.* 2004; Pages *et al.* 2005; Galon *et al.* 2006; Laghi *et al.* 2009; Roxburgh *et al.* 2009; Salama *et al.* 2009; Correale *et al.* 2010; Deschoolmeester *et al.* 2010; Frey *et al.* 2010; Lee *et al.* 2010; Mlecnik *et al.* 2011), tumours with high densities of CD3+ ($P=0.02$), CD8+ ($P<0.01$), CD8+GZMB+ ($P<0.01$) and FOXP3+ ($P<0.01$) cells were associated with significantly improved patient survival. In multivariate analysis, only tumour stage and histological grade were significantly associated with survival (Table 5).

Table 4. Univariate survival analysis for clinicopathological features and T-lymphocyte subtypes for patients treated with surgery alone.

Feature	HR	95% CI	*P*
Clinicopathological Features			
Gender (female *vs* male)	1.26	0.90-1.76	0.18
Age (≥60 yrs *vs* <60 yrs)	1.35	0.86-2.11	0.19
Ethnicity (Chinese *vs* non-Chinese)	1.11	0.63-1.96	0.72
Stage (III & IV *vs* I & II)	5.11	3.60-7.26	<0.01
Site (distal & rectal *vs* proximal)	0.96	0.68-1.36	0.82
Tumour size (≥5 *vs* <5cm)	0.92	0.65-1.29	0.62
Grade (poor *vs* well-moderate)	2.78	1.75-4.39	<0.01
Vascular invasion (yes *vs* no)	2.40	1.42-4.07	<0.01
Lymphatic invasion (yes *vs* no)	2.85	1.63-4.97	<0.01
Perineural invasion (yes *vs* no)	2.56	1.35-4.89	<0.01
Perforation (yes *vs* no)	1.74	0.88-3.41	0.11
MSI status (MSI vs MSS)	0.78	0.34-1.78	0.55
T-lymphocyte subtypes			
CD3+ (high *vs* low)	0.67	0.48-0.94	0.02
CD8+ (high *vs* low)	0.58	0.42-0.81	<0.01
CD8+GZMB+ (high *vs* low)	0.57	0.41-0.80	<0.01
FOXP3+ (high *vs* low)	0.58	0.42-0.80	<0.01

CI: confidence intervals; HR: hazard risk ratio

Table 5. Multivariate survival analysis for clinicopathological features and T-lymphocyte subtypes for patients treated with surgery alone.

Feature	HR	95% CI	*P*
Stage (III & IV *vs* I & II)	4.29	2.95-6.25	<0.01
Grade (poor *vs* well-moderate)	2.21	1.31-3.74	<0.01
Vascular invasion (yes *vs* no)	1.34	0.77-2.32	0.31
Lymphatic invasion (yes *vs* no)	1.23	0.64-2.35	0.54
Perineural invasion (yes *vs* no)	1.43	0.72-2.81	0.30
CD3+ (high *vs* low)	1.30	0.81-2.08	0.28
CD8+ (high *vs* low)	0.67	0.37-1.21	0.18
CD8+GZMB+ (high *vs* low)	0.93	0.51-1.71	0.82
FOXP3+ (high *vs* low)	0.86	0.59-1.26	0.45

CI: confidence intervals; HR: hazard risk ratio

6.3 Predictive Significance of T-lymphocyte Subtype Density

Due to the irregular application of chemotherapy in stage II and IV patients, the predictive significance of clinicopathological features and of T-lymphocyte subtype densities was investigated in the stage III patient subgroup. Overall, patients treated with 5-FU chemotherapy showed significantly better survival compared to those treated by surgery alone ($P<0.01$, Table 6). Of the clinicopathological factors, significant differences in survival between 5-FU-treated and non-treated patients were observed for females, older patients and those with distal colon or rectal tumours (all $P<0.01$). A significantly better survival for 5-FU-treated compared to non-treated patients was also observed in subgroups of patients with well/moderate differentiated ($P<0.01$), poorly differentiated ($P=0.03$), and MSS ($P=0.02$) tumours. Patients with high densities of CD3+, CD8+ or CD8+GZMB+ cells had a significantly better survival if treated with chemotherapy compared to surgery alone (hazard ratio (HR) 0.18, 0.26 and 0.26, respectively; each $P<0.01$). Patients with low densities of these cells treated with 5-FU also had a better survival than those without treatment, although to a lesser degree (HR 0.55, 0.51 and 0.51, respectively; $P=0.11$, 0.05 and 0.06, respectively). The difference in the HRs between high and low density groups was significant for CD3+ T cells ($P=0.032$). Patients with high and low FOXP3+ cell densities treated with 5-FU had a better survival than those untreated to a similar degree (HR 0.41 and 0.43; $P=0.11$ and $P=0.01$, respectively).

Table 6. Predictive significance of clinicopathological features and of T-lymphocyte subtype densities in stage III CRC patients.

Feature (N1, N2)*	HR**	95% CI	*P*	*P* (ΔHR)[#]
All cases (48, 71)	0.37	0.21-0.65	<0.01	>0.05
Clinicopathological				
Gender: Male (22, 37)	0.63	0.27-1.46	0.28	
Gender: Female (26, 34)	0.24	0.11-0.53	<0.01	0.05
Age<60 years (4, 36)	0.98	0.13-7.67	0.98	
Age≥60 years (44, 35)	0.42	0.22-0.80	<0.01	0.22
Grade: Well & Moderate (41, 64)	0.42	0.23-0.76	<0.01	
Grade: Poor (7, 7)	0.13	0.02-0.78	0.03	0.12
Tumour Site: Proximal (19, 29)	0.42	0.17-1.03	0.06	
Tumour Site: Distal & rectal (29, 42)	0.34	0.17-0.69	<0.01	0.36
MSI: Absence (22, 39)	0.39	0.18-0.84	0.02	
MSI: Presence (5, 6)[a]	0.004	<0.001-6316.6	0.45	ND
T-lymphocyte subtypes				
CD3+ High (20, 32)	0.18	0.07-0.46	<0.01	
CD3+ Low (28, 39)	0.55	0.27-1.14	0.11	0.03
CD8+ High (18, 33)	0.26	0.10-0.68	<0.01	
CD8+ Low (30, 38)	0.51	0.26-1.00	0.05	0.13
CD8+GZMB+ High (19, 37)	0.26	0.11-0.64	<0.01	
CD8+GZMB+ Low (29, 34)	0.51	0.25-1.03	0.06	0.12
FOXP3+ High (17, 38)	0.41	0.14-1.23	0.11	
FOXP3+ Low (31, 33)	0.43	0.22-0.83	0.01	0.47

*N1, number of patients treated with surgery alone; N2, number of patients treated with adjuvant 5-fluorouracil-based chemotherapy.

**Hazard ratios for the survival of patients who received 5-fluorouracil-based chemotherapy compared to those treated by surgery alone.

[#]Comparisons of hazard ratio for all parameters.

[a]*P* value for hazard ratio difference is not determined due to low number of cases.

6.4 Discussion

A large and growing body of evidence has established that high densities of TILS are prognostic for good outcome in human CRC (Ropponen *et al.* 1997; Naito *et al.* 1998; Guidoboni *et al.* 2001; Chiba *et al.* 2004; Menon *et al.* 2004; Prall *et al.* 2004; Pages *et al.* 2005; Galon *et al.* 2006; Laghi *et al.* 2009; Roxburgh *et al.* 2009; Salama *et al.* 2009; Correale *et al.* 2010; Deschoolmeester *et al.* 2010 ; Frey *et al.* 2010; Lee *et al.* 2010 ; Mlecnik *et al.* 2011). These studies have demonstrated a role for the adaptive immune response in influencing the clinical behavior of CRC (Galon *et al.* 2006), possibly by preventing the early stages of metastatic invasion (Pages *et al.* 2005). The results of the present work confirm that high densities of tumour-infiltrating T-lymphocyte subtypes are associated with early tumour stage (Table 3) and with better patient survival (Table 4). However, the major and novel finding of this study relates to the predictive significance of T-lymphocyte subtypes (Table 6). The current results expand upon earlier work on the predictive value of TILS in CRC (Morris *et al.* 2008; Halama *et al.* 2009) and breast cancer (Ladoire *et al.* 2008; Denkert *et al.* 2010) by investigating the predictive significance of different T-lymphocyte subtypes.

Similar to the results of Galon *et al*. (Galon *et al.* 2006), the densities of CD3+, CD8+ and CD8+GZMB+ cells were found to be significantly correlated (Figure 6). In agreement with Salama *et al* (Salama *et al.* 2009), positive correlations were also observed between the density of FOXP3+ immune cells and the other subtypes. It has been proposed that tumour-infiltrating FOXP3+ T regulatory cells in CRC are

indicative of homeostatic control of a robust immune response, rather than being merely immunosuppressive (Matera *et al.* 2010). This could explain why the density of FOXP3+ cells was positively associated with the densities of CD8+ and CD8+GZMB+ cytotoxic cells.

Further evidence for the validity of our CRC cohort was derived from the observation that the pathological features of tumour cell invasion into vascular, lymphatic and perineural spaces were each associated with poor prognosis (Table 4). In addition, the better prognosis associated with high densities of the four immune cell subtypes investigated here (Table 5) concur with the results of many other studies (Ropponen *et al.* 1997; Naito *et al.* 1998; Guidoboni *et al.* 2001; Chiba *et al.* 2004; Menon *et al.* 2004; Prall *et al.* 2004; Pages *et al.* 2005; Galon *et al.* 2006; Laghi *et al.* 2009; Roxburgh *et al.* 2009; Salama *et al.* 2009; Correale *et al.* 2010; Deschoolmeester *et al.* 2010; Frey *et al.* 2010; Lee *et al.* 2010). Although the T-lymphocyte subtype densities were not independent prognostic factors in the present series of CRC with mixed stage, these markers deserve further investigation in large, well characterized cohorts of early stage (node-negative) CRC. There is an urgent need for more accurate and robust prognostic markers in stage II CRC. This could be met by semi-automated, quantitative evaluation of T-lymphocyte subtype density, possibly in combination with careful assessment of tumour cell invasion into lymphovascular and perineural spaces (Salama *et al.* 2009).

By comparing the survival of stage III CRC patients treated with or without 5-FU-based chemotherapy, the predictive significance of various factors including immune

cell densities were estimated. In agreement with several previous studies (Elsaleh *et al.* 2000; Morris *et al.* 2007; Dahl *et al.* 2009), the survival of patients treated with 5-FU compared to those untreated was greater for female than male patients (HR=0.24, 0.63 respectively, $P<0.01$, 0.28 respectively; Table 6). A significantly better survival for patients treated with chemotherapy was also seen for older patients and those with left-sided tumours. Patients with high densities of tumour-infiltrating CD3+, CD8+ and CD8+GZMB+ cells (HR=0.18, 0.26, 0.26, respectively; all $P<0.01$) had a better survival from 5-FU chemotherapy than patients with low densities (HR=0.55, 0.51, 0.51, respectively, $P=0.11$, 0.05, 0.06, respectively), with the difference for CD3+ being significant ($P=0.032$). These results should be interpreted with caution because of the small sample sizes for several subgroups and the lack of randomization for chemotherapy. Nevertheless, they suggest that patients with high densities of TILS gain a greater survival advantage from 5-FU-based chemotherapy over those with low densities. This observation warrants further investigation in larger cohorts from randomized clinical trials of other cytotoxic and targeted treatment regimens.

A potential confounder of interest to the prognostic and predictive significance of immune cell subtypes is MSI status, which has been associated with a higher degree of lymphocytic infiltration (Guidoboni *et al.* 2001; Prall *et al.* 2004; Salama *et al.* 2009)., improved prognosis (Popat and Houlston 2005; Sinicrope *et al.* 2006) and a predictive significance (Barratt *et al.* 2002; Ribic *et al.* 2003; Carethers *et al.* 2004) in prior studies. Due to the incomplete availability of tumour blocks, only 287 cases could be examined for MSI status in this study. Some 31 (11%) tumours had MSI, which is a frequency similar to that observed in other East Asian CRC patient

series (Meng *et al.* 2007; Jeon *et al.* 2008). Also consistent with prior findings was the association between MSI and high CD8+ (P=0.04) and high CD8+GZMB+ (P<0.01) T cell densities (Guidoboni *et al.* 2001; Prall *et al.* 2004; Salama *et al.* 2009), supporting the validity of the MSI analysis. However, MSI was not significantly associated with survival in untreated patients (Table 4), which is inconsistent with prior findings (Popat and Houlston 2005; Sinicrope *et al.* 2006). In the analysis of predictive significance, MSS patients treated with chemotherapy had a significantly better survival than those untreated (HR=0.39; p=0.02, Table 6), consistent with the findings of Carethers *et al.* (Carethers *et al.* 2004), However, the analysis of the predictive significance of MSI status could not be completed due the small sample size (n=11) of the MSI patients (Table 6). The only appropriate conclusion from these results may be that the initially small number of MSI cases (n=31), together with their further subgroup analysis preclude adequate assessment of prognostic (in untreated cases only), and predictive (in stage 3 cases according to treatment status) significance.

The present study confirms and extends the results of Morris *et al.* (Morris *et al.* 2008) who found that stage III CRC patients whose tumours showed a dense lymphocytic infiltrate, as determined from the pathology report, gained more survival advantage from adjuvant 5-FU chemotherapy than patients without this feature. It is presently unclear why CRC with a strong TIL reaction are apparently more responsive to 5-FU chemotherapy. One explanation may be that the release of soluble cytokines following chemotherapy-induced tumour cell death activates cytotoxic T-lymphocytes (Lake and Robinson 2005; Zitvogel *et al.* 2008). In this case, it might

be expected that tumours showing evidence of a strong anti-tumour immune response prior to treatment would be more sensitive to chemotherapy. Recent work with an animal model suggests the anti-tumour effect of 5-FU could in part be mediated by its selective cytotoxic activity against myeloid-derived suppressor cells (MDSC) in the tumour bed (Vincent *et al.* 2010). MDSC are well known inhibitors of CD8+ T-lymphocyte activation (Gabrilovich and Nagaraj 2009) and hence it would be interesting to quantify the prognostic and predictive values of MDSC cells in future work on CRC.

It was recently reported that a high density of tumour-infiltrating FOXP3+ immune cells correlated with good prognosis in colon cancer patients undergoing chemotherapy or chemo-immunotherapy (Correale *et al.* 2010). However, the lack of an arm without treatment in that study meant that it was impossible to estimate the predictive value of FOXP3+ cell density. The ratio of intraepithelial immune cells to total number of TILS staining for CD3, CD8 and GZMB was found to be predictive of response to chemotherapy in metastatic CRC (Halama *et al.* 2009), suggesting that immune cell localization as well as density may be important in determining chemo-responsiveness. However, it was unclear from this study whether the individual T-lymphocyte subtype densities alone were predictive of response.

Two studies of neoadjuvant chemotherapy in human breast cancer have provided clear evidence of the predictive significance of tumour-infiltrating immune cells. In the first, the number of intra-tumoural lymphocytes in pretreatment breast tumours was a significant and independent predictor for complete pathological response; with

response rates of 40-42% and 3-7% for high and low cell density tumours, respectively (Denkert *et al.* 2010). The second study reported that complete pathological response correlated with a decreased number of tumour-infiltrating FOXP3+ cells (Ladoire *et al.* 2008). Treatment-related changes in the density of tumour-infiltrating T-lymphocyte subtypes and their predictive significance for pathological response could not be assessed in the current study of CRC treated with adjuvant chemotherapy. However, this would be an interesting area for further investigation in relation to the neoadjuvant treatment of rectal cancers.

In conclusion, the results of our study confirm that high densities of tumour-infiltrating CD3+, CD8+, CD8+GZMB+ and FOXP3+ cells are associated with good prognosis in CRC patients. Our results also suggest that patients with high densities of TILS; in particular CD3+ T cells, may have a better response to 5-FU-based chemotherapy compared to patients with low densities. Further work is required to elucidate the cellular mechanisms that link a strong TIL reaction to an apparently better response to cytotoxic chemotherapy.

7 INVESTIGATION OF T LYMPHOCYTE DENSITIES IN BLOOD AND TUMOURS, AND GENETIC BASIS

7.1 Introduction

Human tumours often contain infiltrates of immune cells such as T-lymphocytes, dendritic T cells and macrophages (Ohtani 2007). These lymphocytes are known as tumour-infiltrating lymphocytes (TILS) and have been associated with good prognosis in various cancers including CRC (Ropponen *et al.* 1997; Naito *et al.* 1998; Guidoboni *et al.* 2001; Chiba *et al.* 2004; Menon *et al.* 2004; Prall *et al.* 2004; Pages *et al.* 2005; Galon *et al.* 2006; Laghi *et al*. 2009; Roxburgh *et al.* 2009; Salama *et al.* 2009; Correale *et al.* 2010; Deschoolmeester *et al.* 2010; Frey *et al.* 2010; Vincent *et al.* 2010; Mlecnik *et al.* 2011). TILS subtypes can be readily identified by distinctive cellular markers. These include CD4+ effector cells, CD8+ cytotoxic cells and their activated form with Granzyme B (*GZMB*) expression, CD45RO+ antigen sensitized cells and CD4+/CD25+/FOXP3+ regulatory cells (T4regs). Anti-tumour activity has been attributed to the first four cell types, whereas T4regs are postulated to exert a suppressive effect on these T cell activities (Clarke *et al.* 2006).

TILS can cause T cell-mediated adaptive immune response which target tumour cells expressing tumour-associated antigens leading to inhibition of tumour growth and progression (Shankaran *et al.* 2001). Several studies have reported that elevated levels

of immune cells are observed in CRC patients in comparison with normal healthy donors (Clarke *et al.* 2006; Ling *et al.* 2007; Chaput *et al.* 2009). This has suggested that the activation of immune response is more likely to be systemic than simply localized in tumours. Ahmadzadeh *et al.* compared the functional and phenotypic characteristics of T4reg cells in tumours and blood of melanoma patients. The blood T4reg cells showed identical expression of surface markers as the tumour T4regs. When these cells were exposed to stimulants, both T4regs also responded in a similar way based on the detection of the cytokines produced, showing that the T4reg cells from blood and tissue are highly similar (Ahmadzadeh *et al.* 2008). Similar findings were also reported in CRC patients (Ling *et al.* 2007). The results of these studies show circulating immune cells share many similarities with intra-tumoural lymphocytes with respect to their phenotypic features and functions. However, it remains unclear if individuals with high concentrations of circulating T-lymphocytes are the individuals with a TILS phenotype.

Evaluation of TILS subtype densities in three independent cohorts revealed that the intensity of the immune reaction at the tumour site can be a determinant of patients' clinical outcomes (Galon *et al.* 2006). Tumours with a strong adaptive immune reaction correlated with highly favorable prognosis whereas a low density of adaptive immune cells correlated with a very poor prognosis even in patients with minimal tumour invasion (Galon *et al.* 2007). However, the underlying mechanisms for the presence of high or low adaptive immune responses are unclear.

Several SNPs (single nucleotide polymorphism) of T cell markers were reported to

have influence on the levels of their corresponding T cell subtypes (Table 7). The presence of the SNP ss46531523 in the perforin gene was correlated with fewer perforin+ cells among circulating CD8+ CTL (McIlroy *et al.* 2006). Three SNPs found in the promoter region of *FOXP3* were associated with primary biliary cirrhosis, which may imply an inadequate Treg function in suppressing the autoimmune response to the liver causing bile duct destruction (Hanel *et al.* 2011). Furthermore, a non-synonymous SNP (55Q/Q) in *GZMB* was also reported to be associated with increased *in-vitro* expression of Granzyme B (Girnita *et al.* 2009). These findings suggest that the intensity of an individual's immune response could be an early manifestation of genetic differences.

The objectives in this project are as follows: (1) to characterize the correlation between the densities of T cell subtypes in the peripheral blood and primary tumours of CRC patients; (2) to examine the correlation between genetic variants in *CD8a*, *GZMB*, *FOXP3* and blood and tumour T cell densities.

Table 7. Overview of Studies on SNP Analysis in T Cell Representative Markers.

Gene	Sample	SNPs	Outcome	References
Perforin	14 CTL cell lines generated from HIV+ patients	• 63 A/G (ss46531524) • 112 A/G (rs10999428) • 1012 C/T (ss46531523)	• The presence of the 1012T genotype correlated with fewer perforin+ cells among circulating CD8+ CTL.	Mcllroy *et al.* 2006
GZMB	396 pediatric heart transplant ; recipients 52 healthy controls	• 55 Q/R (rs 8192917) • -295 A/G (rs 7144366)	• 55 Q/Q genotype is associated with increased *in vitro* expression of Granzyme B.	Girnita *et al.* 2009
FOXP3	Luciferase assays	• -794 C/G (ss270137548) • -738 C/T (rs11091253) • -540 C/T (ss270137549)	• construct −794 C/G showed a significant increased activity than major allele. • promoter variant with two SNPs −738 C/T and −540 C/T displayed a significantly higher activity compared with major allele.	Hanel *et al.* 2011
FOXP3	120 SLE patients; 160 matched controls	• -2383 C/T (rs 3761549) • -3281 C/A (rs 3761548)	• -2383T/-3281A allele was associated with increased risk of SLE.	Lan *et al.* 2010
FOXP3	93 Crohn's diseases; 82 with primary biliary cirrhosis (PBC).	• -605 4 -/ATT (rs590234) • -3279 A/C (rs3761548) • -924 A/G (rs2232364) • IVS9+459 T/C (rs2280883)	• IVS9+459 associated with risk of primary biliary cirrhosis.	Park *et al.* 2005

7.2 Results

7.2.1 Correlation of T Cell Subtype Densities in Blood and Tumour of CRC Patients

Mononuclear cells extracted from 41 CRC patients' peripheral blood were stained with antibodies of T cell markers and analyzed by flow cytometry. Cells with identical size and complexity were gated and further sorted based on the expression of CD3. T4reg and activated cytotoxic T cells were recognized as CD4+FOXP3+ and CD8+GZMB+ cells, respectively (Figure 7). The distribution of the levels of circulating T cell subtypes is shown in Figure 8. The median values of CD8+, CD8+GZMB+ and CD4+FOXP3+ T cells were 32% (15%-66%), 14% (2%-61%) and 3% (0.04%-9%).

A total of 41 matched tumours were also stained with the same T cell markers as above by IHC (Figure 9). The number of positively stained lymphocytes was counted manually for each regions and the density of lymphocytes for each tumour was then expressed as cells per square millimeter. Median densities of intra-tumoural CD8+cells, CD8+GZMB+ cells and CD4+FoxP3+ cells were 546 cells/mm^2, 186 cells/mm^2 and 86 cells/ mm^2, respectively (Figure 8 D-F).

Figure 10 shows the densities of CD8+, CD8+GZMB+ and CD4+FOXP3+ T cells in tumours were correlated with the levels of their circulating counterparts significantly. (Pearson correlation coefficients r=0.38, r=0.33 and r=0.28, respectively, all $P<0.05$)

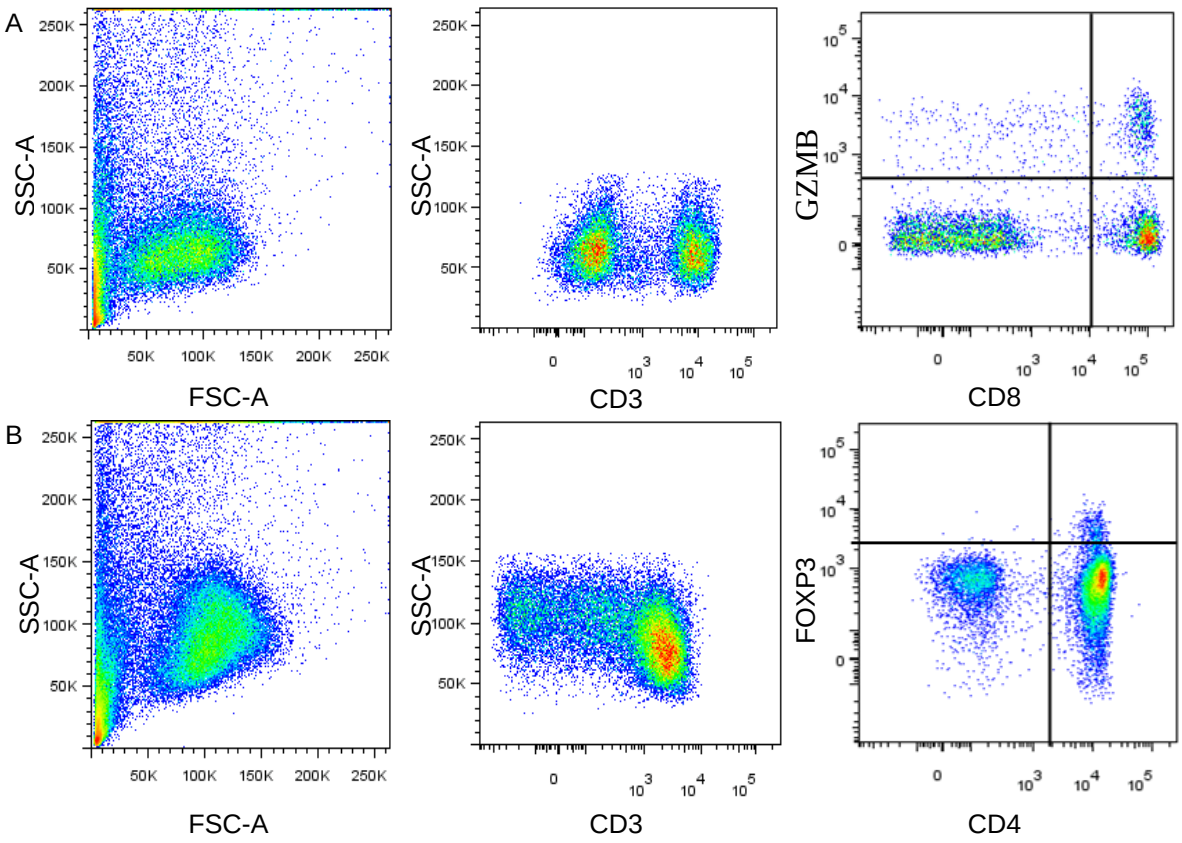

Figure 7. Gating strategies for activated cytotoxic T cells (A) and T4regs (B) using flow cytometry on peripheral blood

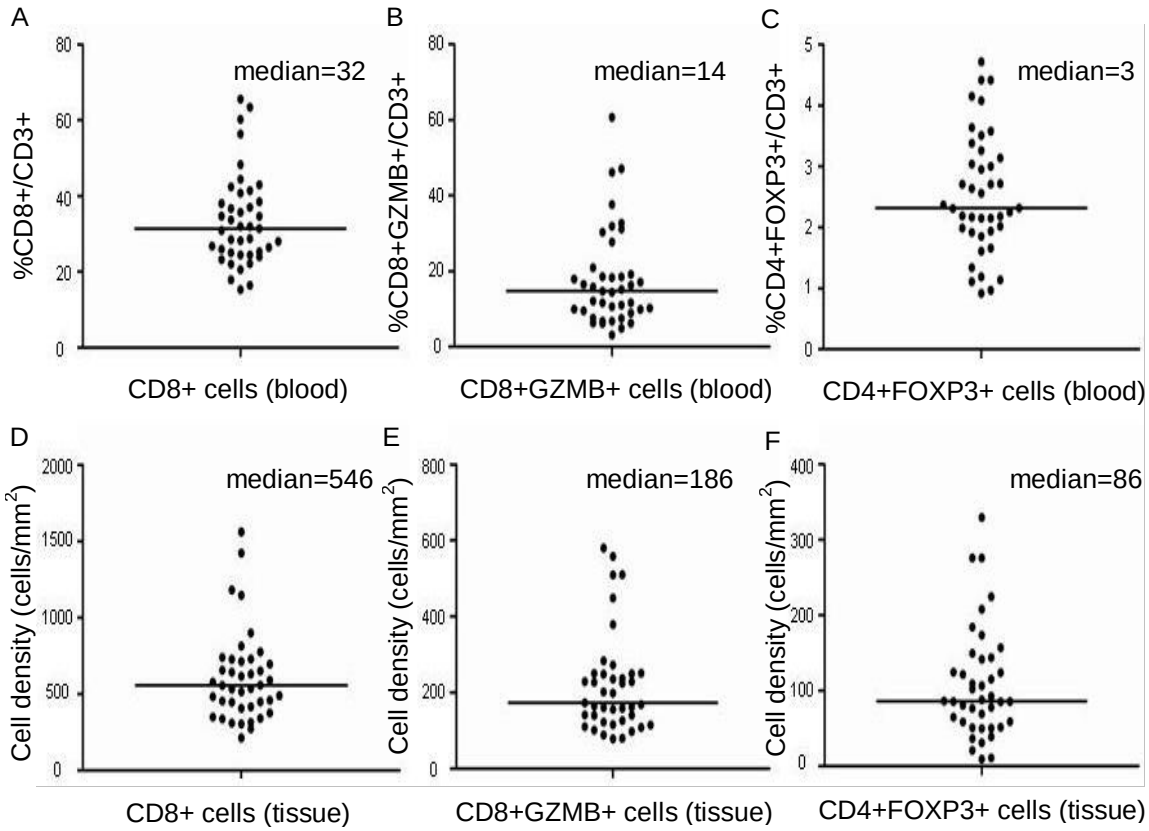

Figure 8. Density of T-lymphocyte subtypes, including CD8+ T cells (A, D), CD8+GZMB+ T cells (B, E) and CD4+FOXP3+ T cells (C, F) observed in 41 paired blood and tumours from CRC patients

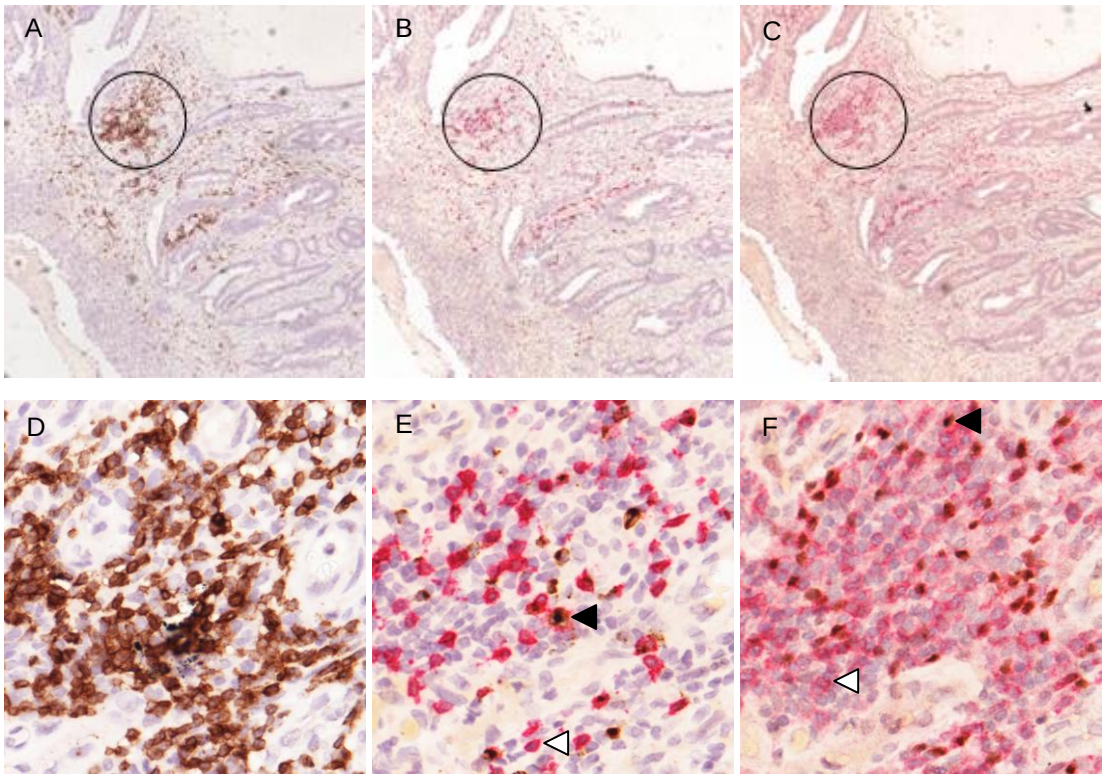

Figure 9. Immunohistochemical stains for CD3 (A), double staining of CD8 and GZMB (B) as well as CD4 and FOXP3 T cells (C) in the same region of a CRC tumour at 100x magnification. Panel below are enlarged images for the black circle regions taken at 400x magnification. The black arrows highlight activated cytotoxic T cells with coexpression of CD8 (red) and GZMB (brown) (E) and T4reg cells with co-expression of CD4 (red) and FOXP3 (brown) (F). The white arrows indicate cytotoxic T cells with no GZMB expression (E) and CD4 T helper cells (F).

Brown and red stains were achieved by incubation with DAB and alkaline phosphatase red respectively; and the nuclei were counterstained with haematoxylin.

7.2.2 Correlation of Gene Expression of T Cell Markers with Their Corresponding T Cell Subtype Levels in Peripheral Blood

The relative gene expression level of *CD8a* showed strong correlation with the proportion of CD8+ T cell in peripheral blood (r=0.48, *P*<0.01). However, there was no significant correlation between gene expression levels and their corresponding blood T cell densities for *GZMB* (r=0.17, *P*=0.30) and *FOXP3* (r=0.19, *P*=0.22), (Figure 11).

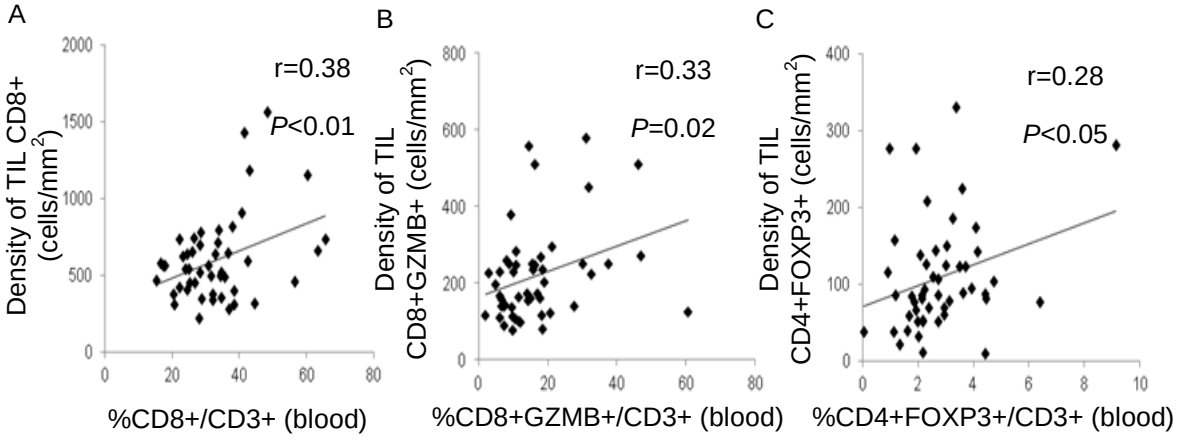

Figure 10. Correlation between densities of intra-tumoural and circulating CD8+ T cells (A), CD8+GZMB+ T cells (B) and CD4+FOXP3+ T cells (C).

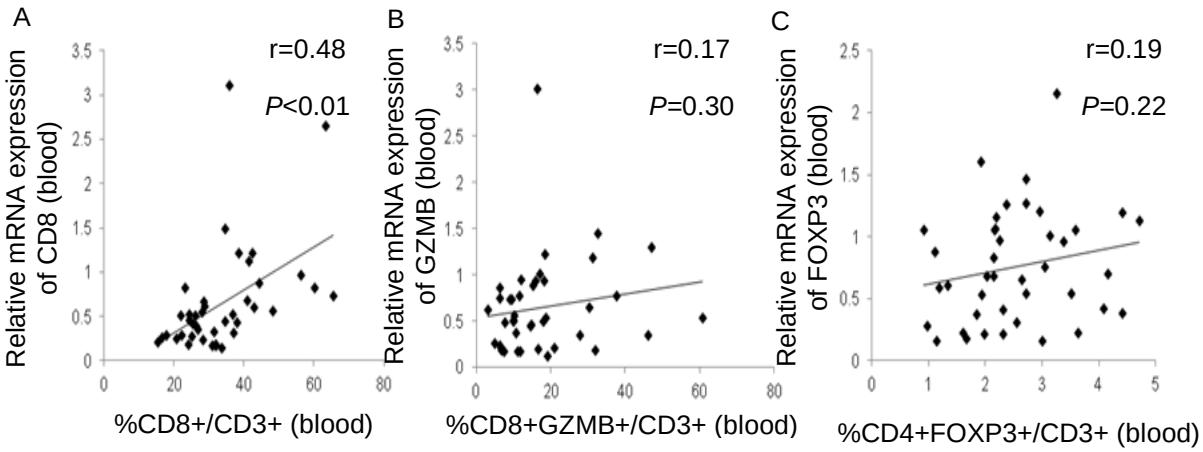

Figure 11. Correlation between gene expression levels of T cell markers: CD8a (A) GZMB (B) and FOXP3 (C) and their corresponding circulating T-lymphocyte subtype levels.

7.2.3 Correlation of Genotypes with Densities of Intra-tumoural and Levels of Circulating T Cell Subtypes

Twelve SNPs from *CD8a*, 22 from *GZMB* and 9 from *FOXP3* were genotyped in 41 CRC patients with blood and tissue densities. The other nine cases were unable to be genotyped due to sample exhaustion. The minor allele frequency (MAF) of these SNPs showed ≥1% in Asian cohorts from dbSNP database (Table 8). The SNPs genotype distributions reported in our study were compared to those of Han Chinese and Japanese cohorts by Chi-square test (Table 9). The majority of distributions were similar between series with differences only observed in 10/39 (25%) of genotypes (Table 9).

Table 10 shows that a total of 11 SNPs from *GZMB* and *FOXP3* were found associated with their respective T cell subtype densities in blood (all $P<0.05$). GZMB SNP rs#8192922, and two SNPs from FOXP3 (rs#3761549 and rs#3761547) were significantly associated with their tumour T cell subtype densities ($P=0.01$, 0.02 and 0.02, respectively). SNP #rs8192922 was the only SNP associated with the CD8+GZMB+ cell densities significantly in tissue and blood (both $P=0.01$). As shown in Figure 12, higher densities of circulating and intra-tumoural CD8+GZMB+ cells were seen in the patients with genotype GG compared to GA carriers. There were no AA individuals observed in this study.

Table 8. Frequencies of SNPs in genes encoding CD8, GZMB and FOXP3.

Gene	SNP ID	Nucleotide change*	Location	Amino Acid Change	Allelic Frequency in Chinese/Asian	
CD8a	rs2944254	G>A	Intron 5		G:0.84	A:0.16
CD8a	rs2367377	A>C	Near 3' UTR		A:0.91	C:0.09
CD8a	rs1515950	G>A	5' UTR		G:0.84	A:0.16
CD8a	rs3020729	T>C	Exon 6	Non coding	T:0.84	C:0.16
CD8a	rs6743139	G>A	5' UTR		G:0.93	A:0.07
CD8a	rs3810831	A>G	5' UTR		A:0.92	G:0.08
CD8a	rs6718678	C>T	Near 3' UTR		C:0.79	T:0.21
CD8a	rs938487	T>C	Near 3' UTR		T:0.81	C:0.19
CD8a	rs2367376	A>G	Near 3' UTR		T:0.81	C:0.19
CD8a	rs1051386	T>C	Exon 6	Non coding	T:0.85	C:0.15
GZMB	rs8192920	G>T	Near 3' UTR		G:0.87	T:0.13
GZMB	rs2273844	G>A	Exon 1	Non coding	G:0.68	A:0.32
GZMB	rs1957528	T>C	5' UTR		T:0.75	C:0.25
GZMB	rs8012137	A>G	Near 3' UTR		A:0.57	G:0.43
GZMB	rs7141106	A>T	5' UTR		A:0.51	T:0.49
GZMB	rs1957527	A>T	5' UTR		A:0.72	T:0.28
GZMB	rs8192916	T>C	5' UTR		T:0.52	C:0.48
GZMB	rs6573910	C>T	Intron 4		C:0.68	T:0.32
GZMB	rs6573913	A>T	5' UTR		A:0.72	T:0.28
GZMB	rs8192919	C>T	Near 3' UTR		C:0.91	T:0.09
GZMB	rs7144366	C>T	5' UTR		C:0.57	T:0.43
GZMB	rs2236338	A>G	Exon 5	Try→His	A:0.67	G:0.33
GZMB	rs8192922	G>A	Near 3' UTR		G:0.82	A:0.18
GZMB	rs10909625	A>G	Exon 3	Lys→Lys	A:0.67	G:0.33
GZMB	rs6573912	T>C	5' UTR		T:0.75	C:0.25
GZMB	rs9671454	C>G	Intron 2		C:0.99	G:0.01
GZMB	rs8192917	A>G	Exon 2	Arg→Gln	A:0.68	G:0.32
GZMB	rs8016685	T>A	Near 3' UTR		T:0.57	A:0.43
GZMB	rs2236337	T>C	Exon 5	Non coding	T:0.66	C:0.34
GZMB	rs762085	A>T	Near 3' UTR		A:0.57	T:0.43
GZMB	rs6573911	C>T	Intron 4		C:0.67	T:0.33
GZMB	rs10873219	G>T	Intron 2		G:0.82	T:0.18
FOXP3	rs3761549	C>T	Intron 1		C:0.77	T:0.23
FOXP3	rs2280883	T>C	Intron 10		T:0.79	C:0.21
FOXP3	rs11091253	C>T	5' UTR		C:0.99	T:0.05
FOXP3	rs3761547	A>G	Intron 1		A:0.77	G:0.23
FOXP3	rs3761548	A>C	Intron 1		A:0.80	C:0.20
FOXP3	rs2232365	A>G	Intron 1		A:0.57	G:0.43
FOXP3	rs12843496	C>T	Intron 1		C:0.99	T:0.01

Table 9. Comparison of SNP frequencies between the NUH cohort and other Asian cohorts.

Gene	SNP ID	Nucleotide Change	dbSNP: Asian Cohorts[#]			NUH Cohort			*P* value
			AA*(%)	AB*(%)	BB*(%)	AA*(%)	AB*(%)	BB*(%)	
CD8a	rs2944254	G>A	56 (68)	26 (32)	0 (0)	11 (27)	29 (71)	1 (2)	**<0.01**
CD8a	rs2367377	A>C	78 (91)	0 (0)	8 (9)	36 (88)	5 (12)	0 (0)	**<0.01**
CD8a	rs1515950	G>A	72 (84)	0 (0)	14 (16)	33 (89)	3 (8)	1 (3)	**<0.01**
CD8a	rs3020729	T>C	58 (69)	26 (31)	0 (0)	27 (66)	11 (27)	3 (7)	**0.04**
CD8a	rs6743139	G>A	74 (86)	12 (14)	0 (0)	34 (83)	6 (15)	1 (2)	0.34
CD8a	rs3810831	A>G	72 (84)	14 (16)	0 (0)	25 (81)	5 (16)	1 (3)	0.25
CD8a	rs6718678	C>T	50 (58)	36 (42)	0 (0)	23 (56)	12 (29)	6 (15)	**<0.01**
CD8a	rs938487	T>C	56 (65)	28 (33)	2 (2)	24 (59)	12 (29)	5 (12)	0.08
CD8a	rs2367376	A>G	59 (65)	30 (33)	2 (2)	24 (59)	12 (29)	5 (12)	0.06
CD8a	rs1051386	T>C	60 (70)	26 (30)	0 (0)	16 (52)	8 (26)	7 (23)	**<0.01**
GZMB	rs8192920	G>T	66 (77)	18 (21)	2 (2)	25 (61)	13 (32)	3 (7)	0.13
GZMB	rs2273844	G>A	42 (49)	34 (39)	10 (12)	20 (49)	16 (39)	5 (12)	1.00
GZMB	rs1957528	T>C	48 (57)	30 (36)	6 (7)	19 (46)	18 (44)	4 (10)	0.52
GZMB	rs8012137	A>G	34 (38)	34 (38)	22 (24)	12 (44)	11 (41)	4 (15)	0.56
GZMB	rs7141106	A>T	28 (31)	36 (40)	26 (29)	7 (17)	19 (46)	15 (37)	0.24
GZMB	rs1957527	A>T	48 (53)	34 (38)	8 (9)	19 (46)	18 (44)	4 (10)	0.76
GZMB	rs8192916	T>C	24 (28)	42 (49)	20 (23)	6 (15)	18 (45)	16 (40)	0.10
GZMB	rs6573910	C>T	42 (49)	34 (39)	10 (12)	20 (49)	15 (37)	6 (15)	0.88
GZMB	rs6573913	A>T	48 (53)	34 (38)	8 (9)	20 (50)	16 (40)	4 (10)	0.94
GZMB	rs8192919	C>T	40 (83)	8 (17)	0 (0)	26 (63)	13 (32)	2 (5)	0.06
GZMB	rs7144366	C>T	26 (30)	46 (54)	14 (16)	13 (32)	22 (54)	6 (15)	0.97
GZMB	rs2236338	A>G	40 (46)	36 (42)	10 (12	20 (50)	14 (35)	6 (15)	0.73
GZMB	rs8192922	G>A	62 (70)	22 (25)	4 (5)	36 (88)	5 (12)	0 (0)	0.08
GZMB	rs10909625	A>G	42 (47)	36 (40)	12 (13)	20 (49)	15 (37)	6 (15)	0.93
GZMB	rs6573912	T>C	48 (57)	30 (36)	6 (7)	20 (49)	17 (41)	4 (10)	0.66
GZMB	rs9671454	C>G	86 (98)	2 (2)	0 (0)	4 (10)	15 (37)	22 (54)	**<0.01**
GZMB	rs8192917	A>G	42 (49)	34 (39)	10 (12)	0 (0)	18 (45)	22 (55)	**<0.01**
GZMB	rs8016685	T>A	34 (38)	34 (38)	22 (24)	14 (34)	20 (49)	7 (17)	0.45
GZMB	rs2236337	T>C	41 (45)	38 (42)	12 (13)	20 (49)	15 (37)	6 (15)	0.85

GZMB	rs762085	A>T	34 (38)	34 (38)	22 (24)	17 (41)	17 (41)	7 (17)	0.64
GZMB	rs6573911	C>T	42 (47)	36 (40)	12 (13)	20 (49)	15 (37)	6 (15)	0.93
GZMB	rs10873219	G>T	60 (70)	22 (25)	4 (5)	22 (54)	15 (37)	4 (10)	0.18
FOXP3	rs3761549	C>T	60 (70)	12 (14)	14 (16)	22 (76)	2 (7)	5 (17)	0.60
FOXP3	rs2280883	T>C	58 (71)	14 (17)	10 (12)	32 (78)	5 (12)	4 (10)	0.68
FOXP3	rs11091253	C>T	170 (99)	2 (1)	0 (0)	38 (93)	2 (5)	1 (2)	**0.03**
FOXP3	rs3761547	A>G	60 (70)	12 (14)	14 (16)	0 (0)	32 (80)	8 (20)	**<0.01**
FOXP3	rs3761548	A>C	60 (70)	18 (21)	8 (9)	32 (78)	5 (12)	4 (10)	0.49
FOXP3	rs2232365	A>G	42 (49)	14 (16)	30 (35)	21 (51)	7 (17)	13 (32)	0.94
FOXP3	rs12843496	C>T	168 (99)	0 (0)	2 (1)	41 (100)	0 (0)	0 (0)	0.49

*A: Major allele, B: Minor allele

[#]Han Chinese and Japanese cohorts

Table 10. Correlations of T cell marker SNP frequencies with T cell subtype densities in blood and tissue

Gene	SNP ID	Nucleotide change	Blood (Proportion)*	Tissue (Density)*
CD8a	rs2944254	G>A	0.48	0.10
CD8a	rs2367377	A>C	0.59	0.59
CD8a	rs1515950	G>A	0.37	0.05
CD8a	rs3020729	T>C	0.80	0.35
CD8a	rs6743139	G>A	0.51	0.34
CD8a	rs3810831	A>G	0.12	0.44
CD8a	rs6718678	C>T	0.60	0.30
CD8a	rs938487	T>C	0.20	0.71
CD8a	rs2367376	A>G	0.28	0.71
CD8a	rs1051386	T>C	0.95	0.25
GZMB	rs8192920	G>T	0.09	0.81
GZMB	rs2273844	G>A	**0.02**	0.22
GZMB	rs1957528	T>C	0.10	0.88
GZMB	rs8012137	A>G	0.57	0.77
GZMB	rs7141106	A>T	**0.02**	0.36
GZMB	rs1957527	A>T	0.10	0.88
GZMB	rs8192916	T>C	0.04	0.39
GZMB	rs6573910	C>T	**0.01**	0.21
GZMB	rs6573913	A>T	0.13	1.00
GZMB	rs8192919	C>T	0.23	0.90
GZMB	rs7144366	C>T	0.69	0.65
GZMB	rs2236338	A>G	**0.01**	0.16
GZMB	rs8192922	G>A	**0.01**	**0.01**
GZMB	rs10909625	A>G	**0.01**	0.21
GZMB	rs6573912	T>C	0.19	0.98
GZMB	rs9671454	C>G	**0.04**	0.52
GZMB	rs8192917	A>G	1.00	0.34
GZMB	rs8016685	T>A	0.45	0.45
GZMB	rs2236337	T>C	**0.01**	0.21
GZMB	rs762085	A>T	0.39	0.49
GZMB	rs6573911	C>T	**0.01**	0.21
GZMB	rs10873219	G>T	**0.04**	0.52
FOXP3	rs3761549	C>T	0.61	**0.02**
FOXP3	rs2280883	T>C	0.51	0.86
FOXP3	rs11091253	C>T	0.12	0.13
FOXP3	rs3761547	A>G	0.34	**0.02**
FOXP3	rs3761548	A>C	0.51	0.86
FOXP3	rs2232365	A>G	**0.02**	0.20
FOXP3	rs12843496	C>T	NA	NA

***P* value of likelihood ratio

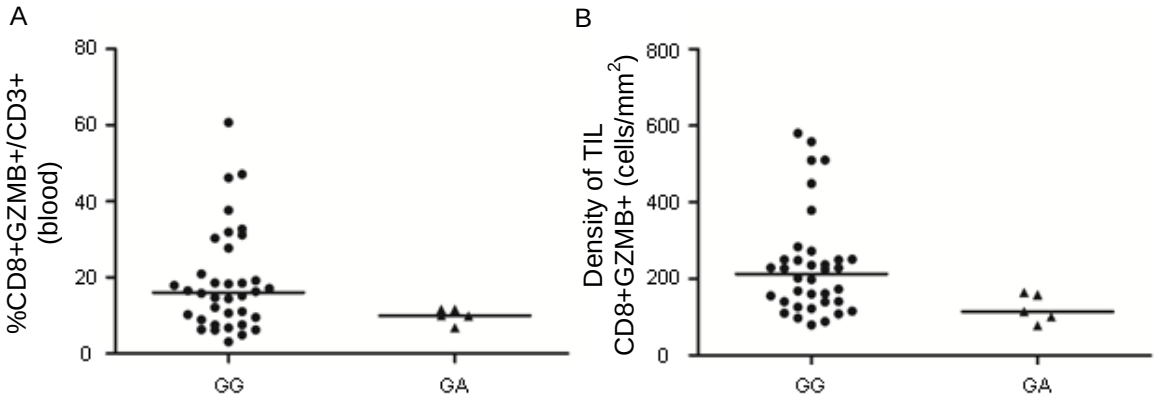

Figure 12. Densities of CD8+GZMB+ T lymphocytes in blood (A) and tumour (B) according to SNP rs 8192922 genotype.

7.3 Discussion

To our knowledge, this is the first study to report on correlations between matched densities of intra-tumoural and circulating T cell subtypes in CRC patients. Our results indicated that individuals with high concentrations of various circulating T cell subtypes may have increased intra-tumoural T cell densities (Figure 10). *In vitro* assays performed by Maccalli *et al*. have shown that reduced cytotoxicity of peripheral blood cells may be due to down-regulated expression of cytotoxic molecules (perforin, granzymeB and FasL) in patients with cancer (Maccalli *et al.* 2008). These findings suggest that the presence of immune cell subsets in blood correlate with their counterparts in tumour sites as a consequence of a global anti-tumour immune response in the host.

The prognostic and predictive values of TILS subtypes in CRC described in Chapter 3, implied that their circulating counterparts may be useful for predicting the clinical outcome of this disease. In the phase II trial of GOLFIG-1 chemoimmunotherapy (combination of gemcitabine, oxaliplatin, levofolinic acid, 5-fluorouracil, granulocyte macrophage colony-stimulating factor and interleukin 2), CRC patients who had an increase in CTLs, memory T cells and depletion of T4regs had a prolonged time to disease progression and overall survival (Correale *et al.* 2008). This implied that the evaluation of circulating T cell subtypes may represent a less invasive method to use to predict prognosis and possibly also the response to treatment. However, further validation by testing the association of patient survival and chemotherapy response with blood cell levels is required in order to support the notion above.

Gene expression level of *CD8a* correlated significantly ($r=0.48$, $P<0.01$) with CD8+ T cells density in the blood. However, no significant correlations were found for both

GZMB and *FOXP3* genes. Protein is the downstream product of mRNA, so one might assume that there should be some degree of correlation between the mRNA and protein (Greenbaum *et al.* 2003). One of the earliest analyses of correlation was performed on 19 proteins in the human liver. A positive correlation of 0.48 was reported by Anderson and Seilhamer (Anderson and Seilhamer 1997). Orntoft *et al.* found highly significant correlations in human carcinomas when looking at the changes in mRNA and protein expression level (Orntoft *et al.* 2002). In contrast, another two studies on prostate and lung cancers reported either no significant correlation, or that a significant correlation is only found in a small subset of the proteins (Chen *et al.* 2002; Lichtinghagen *et al.* 2002). Therefore, the relationship between mRNA and protein expression is still debatable. There are several other possible reasons for the poor correlations reported for *GZMB* and *FOXP3* in our cohort. Firstly, GZMB and FOXP3 are not expressed exclusively in CD8+ and CD4+ T cells. GZMB have also been found to be expressed in other immune cells as well as in normal cells of non-haematopoietic origin such as chodrocytes, keratinocytes, type II pneumocyte, Sertoli cells, primary spermatocytes, cells of granulose and syncytiotrophoblast (Rousalova and Krepela). A small population of CD8+ cells was also identified as FOXP3+ in CRC patients' blood (Chaput *et al.* 2009). Secondly, the involvement of complicated and varied post-transcriptional mechanisms during translation could also account for a lack of correlation (reviewed in (Mansfield and Keene 2009)). In addition, protein may differ substantially in their *in vivo* half lives, affecting their accurate measurement (Glickman and Ciechanover 2002).

A high concordance in the distribution of SNPs genotypes was observed between our cohort and other East Asia populations (Table 9). However, 26% of the SNPs showed

significant differences. The majority of our cohort was Chinese (80%) and they are descendants mainly from Southern provinces of China. Although they might share common ancestral backgrounds with the northern Han Chinese in China, these two populations are genetically varied to some extent due to geographically distinct origin (Teo *et al.* 2009). Another possibility for the differences observed could also be due to analytical methods used for SNPs detection.

Among the 39 SNPs that have examined, *GZMB* SNP #rs8192922 was significantly associated with the density of CD8+GZMB+ T cells in both tissue and blood of CRC patients. This SNP is located downstream of *GZMB* gene transcription end site which is 403 base pairs away and seems to be under a selection pressure, since it is highly conserved across species including gorilla, orangutan, rhesus, marmoset, cow and dog (Figure 13). A highly conserved sequence is more likely to carry with it an important biological role (Levy *et al.* 2001). Regions of conserved non-coding sequence, identified by using comparative sequence analysis methods between mouse and human genomic sequences, were reported to play a role in transcriptional regulation. These suggest that *GZMB* SNP #rs8192922 may be localized at the distal enhancer region which could possibly influence transcription efficiency of *GZMB* gene (Birney *et al.* 2007).

In summary, we found positive correlations between the levels of circulating T cell subtypes with the densities of their corresponding intra-tumoural T cell subtype. The mRNA expression of *CD8a* but not *GZMB* and *FOXP3* correlated with the density of their respective cells. The SNP analysis revealed that CRC patients with GG genotype of *GZMB* SNP #rs8192922 have a higher density of CD8+GZMB+ cells than those with the GA genotype. However, the underlying mechanisms for the effect of the SNP

on CD8+GZMB+ T cell densities are unclear. Our data suggest that SNPs could be potential factors to determine host immune response in tumours.

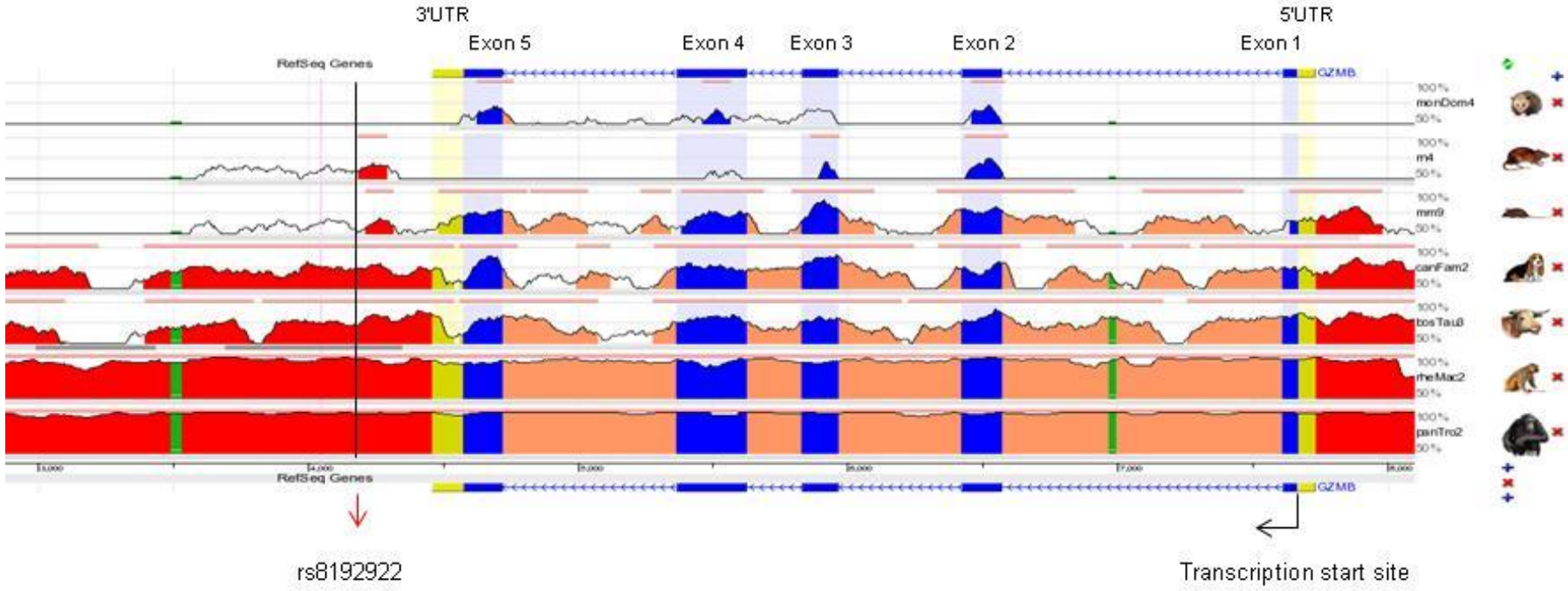

Figure 13. Evolutionary conservation of the GZMB gene (transcribed right to left from the transcription start site indicated at the bottom of the figure). The SNP rs8192922 (indicated by red arrow) is located in a highly conserved region between species.

8 CONCLUSIONS AND FUTURE DIRECTIONS

A minority of colorectal tumours are characterized by the presence of high densities of infiltrating lymphocytes. These have consistently been associated with good patient outcome, with some authors suggesting their prognostic significance can rival or even surpass that of the TNM staging system (Galon *et al.* 2006; Galon *et al.* 2007; Mlecnik *et al.* 2011). The present results confirm the prognostic value of various tumour-infiltrating T cell subtype densities in colorectal cancer, but also suggest they have predictive significance for the response to 5-FU-based chemotherapy. High densities of CD3+, CD8+ and CD8+GZMB+ cells were associated with better survival following adjuvant treatment compared to low densities of these markers. Our findings suggest that immune cell subtype densities should be investigated further as potentially useful clinical biomarkers for response to cytotoxic chemotherapy.

In Chapter 4, we observed positive correlations between the levels of circulating T cell subtypes and the densities of their corresponding intra-tumoural T cells in CRC patients. Taken together, our findings suggest that anti-tumour reactions may not be occurring in tumours only but may be a reflection of a more global immune response. Based on this, we hypothesized that varied intensities of systemic anti-tumour immune response observed in CRC patients could be due to genetic variations in genes encoding immune cell markers. The SNPs analysis on T cell markers showed that CRC patients with GG genotype of *GZMB* SNP rs8192922 have higher density of CD8+GZMB+ cells than those with GA. Further investigation is needed to ascertain the functional significance of GZMB SNP rs8192922.

In summary, our study has confirmed the prognostic and predictive significance of

TILS subtypes. The findings are anticipated to provide new insights on identifying reliable and potential markers that can be included for clinical practice in the future. The assessment of T cell subtypes in blood and tumour also supports the notion that the anti-tumour reaction in CRC patients is a systemic host response. Varied intensities of systemic anti-tumour immune response could be a consequence of polymorphisms that occur in the immune cell genes. Taken together, the results suggest less invasive genetic and/or peripheral blood monitoring of TILS, and consequent prognostication and prediction of 5-FU response in CRC patients, could be feasible. Future work to validate these findings is required.

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